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PREFACE

The search for and the capture of microbiological truths is, in an esoteric fashion, comparable to the painted capture of a mood in a vast, uncharted desert. Even before the paint is dry the light changes, a new scene appears and the painter must begin again. So it is with those of us who would probe each instant of microbial life in order to understand the ordered continuity of the whole. The progression from ephemora to fact, the separation of shadow from the substance is the dream of each microbiologist who, with a palette knife in his hand, attempts to impose upon the canvas of his laboratory, the essence of his own search.

It is the hope of those of us who work within the framework of the *Annual Review of Microbiology* that its pages may serve as a kind of canvas upon which the compositions of the geneticists, the biologists, and the bacteriologists may appear and which may some day merge into a finished portrait. Indeed, the *Review* has tried, over the years, to draw together the facets of this burgeoning science by offering in alternating review the multicolors of an absorbing microscapse. To the artists—these authors—whose names appear in this, Volume 14, and in all past volumes, we offer our profound thanks.

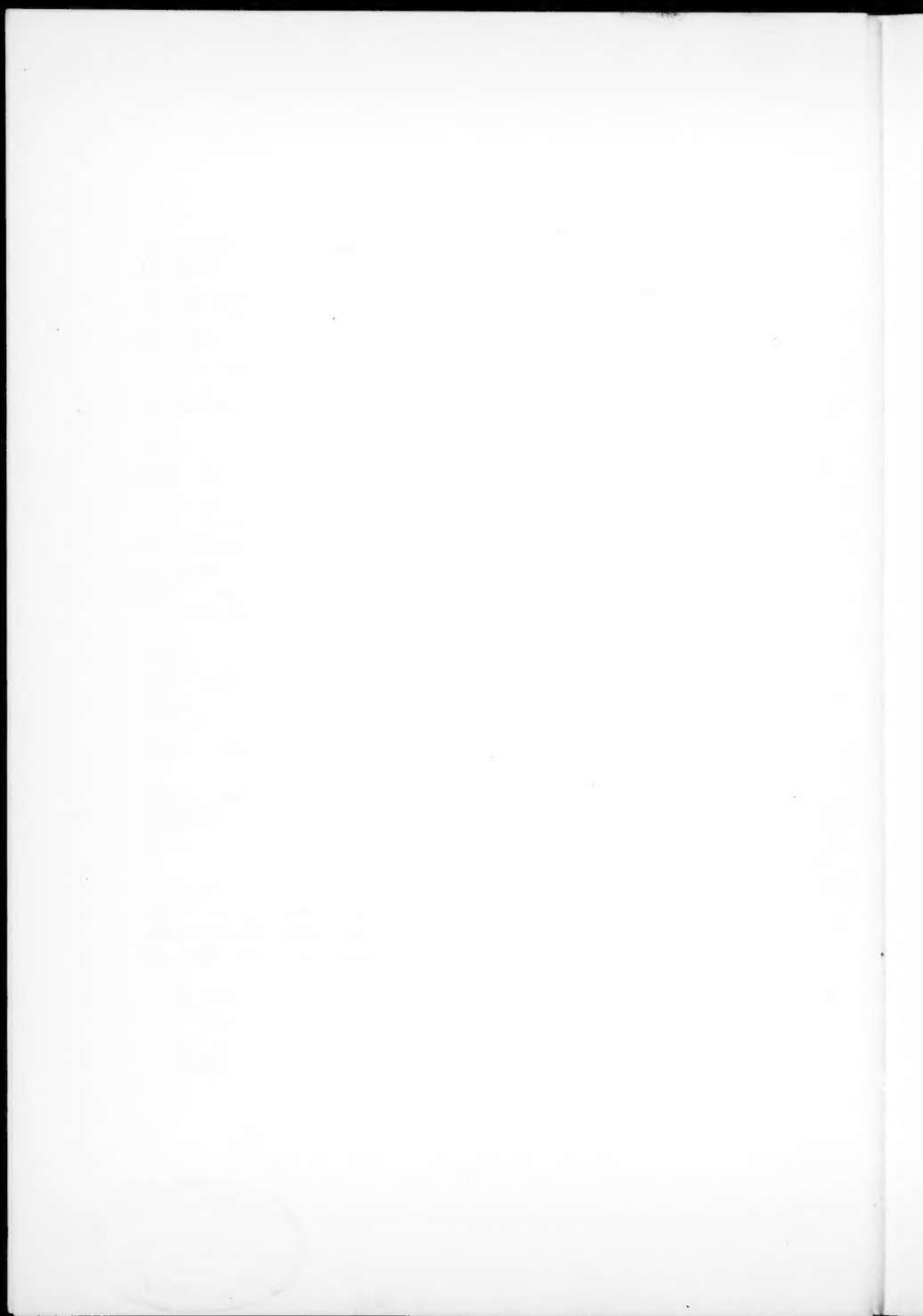
To recently retired S. J. P. Chilton, sincere thanks for having brought much to this and previous volumes. For whatever measure of clear communication is achieved between researcher and reader on the pages of the *Review*, credit must be given, in large part, to the planners—each member of each Editorial Committee. Our latest member, Dr. Paul R. Miller, is welcomed as successor to Dr. Chilton.

No Editorial foreword can be complete without the traditional and appreciative acknowledgment to our Editorial staff and our printers, The Banta Company, of their expert and patient attention to detail, without which none of these volumes would ever be published.

Finally, as a new service to our readers, we are offering with this issue, an index to the reviewers and the reviews that have appeared in all past volumes. In each coming year, five volumes will be cumulatively presented in the hope that they may find usefulness among the palette knife and brush wielders of Microbiology.

C.E.C.	P.R.M.
C.A.E.	S.R.
R.E.H.	M.P.S.
T.L.J.	W.W.U.





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VARIATION IN PHYTOPATHOGENIC FUNGI¹

BY PETER R. DAY

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This review is concerned mainly with variation in the pathogenicity of phytopathogenic fungi. Some recent developments in the genetics of saprophytic fungi which seem particularly relevant are also dealt with. A number of reviews covering variation in phytopathogenic fungi have appeared since that of Christensen *et al.* (20). These include surveys on adaptation in fungi by Christensen & Daly (21), on adaptation of plant pathogen to host by Christensen & DeVay (22), on variability in fungi by Stakman & Christensen (122), and the symposium on the genetic approach to pathogenicity and disease resistance in the volume (60) published to mark the fiftieth anniversary of the foundation of the American Phytopathological Society. Recent reviews of related topics include those of Wheeler (135) on the genetics of fungi, Pontecorvo (103) on the parasexual cycle, Yarwood (141) on obligate parasitism, Barnett (4) on plant disease resistance, and Kiraly & Farkas (75) on biochemical trends in plant pathology.

The author has grouped topics which are genetically related in preference to dealing with the fungi family by family. The most encouraging feature of much recent work in plant pathology is the emphasis placed on the genetic approach. It is hoped that the treatment used here will stimulate this approach still further and cause those who do much valuable work on plant diseases to be more concerned about the genetical basis of their experiments.

In any discussion of plant pathogens the terms, "pathogenicity" and "virulence" are likely to be used to describe ability to incite disease. The distinction between these terms made by Miles (88) has been adopted in this article. Pathogenicity is regarded as a general attribute of a species, while virulence is reserved for pathogenicity of a particular strain of a pathogen in relation to a particular host genotype.

PHYSIOLOGICAL SPECIALISATION

This is a problem encountered in most programmes of breeding for disease resistance. It has been studied in two ways which are of interest here. First, pathologists have defined biotypes which differ in host range. The distribution and number of biotypes of many pathogens are determined by periodic surveys. The results of these surveys are useful to the breeder who screens his breeding stocks for resistance by using the biotypes which most threaten his crops.

Second, pathologists have studied the genetic systems of the pathogens and have shown that they control variation in pathogenicity and virulence. From this approach we may hope to define the limits of variability and to assess the problems the future may hold for breeding resistant host varieties.

¹ The survey of the literature pertaining to this review was concluded in December, 1959

Biotype surveys.—A physiologic race is defined by the host varieties on which it is virulent. It therefore follows that the selection of differential host varieties is a critical factor in defining physiologic races. In practice, the pathologist is most concerned with the physiologic races he finds on important resistant-host varieties and these varieties, or genotypes representative of them, therefore appear among his differential hosts. These and other aspects of the conduct of surveys have recently been considered by Baker & Upadhyaya (3) in Australia, and Simons (118) and Simons & Michel (119) in North America for races of *Puccinia coronata avenae* on oats. Simons advocated the use of a special set of differential host varieties to detect new, potentially dangerous, races soon after they appear. Supplemental differential hosts for identifying new races of wheat leaf rust (*Puccinia recondita*) are also to be adopted (83).

The advantages of using differential hosts with single resistance genes have been discussed by Flor (35) and will be referred to again. The work of Russell & Hooker (110) on the inheritance of resistance to *Puccinia sorghi* in *Zea mays* shows how single-gene differential host varieties may be selected, and describes tests of the genetic relationships among different sources of resistance.

The sampling used in race surveys varies with both pathogen and pathologist. In general, most pathologists favour the use of single spore or single lesion isolates which are multiplied separately and are then inoculated to a set of differential hosts. Where a survey concentrates on detecting the occurrence of new races, bulked samples (consisting of tens or hundreds of lesions from different resistant or susceptible plants) inoculated without multiplication, would seem to be a more economical means of determining whether or not certain virulent genotypes are present. For example, races virulent on several varieties could be detected by looking for lesions on appropriate multiple-gene testers. Such methods have, however, rarely been employed since the samples yield little or no information on the relative proportions of the component biotypes, particularly those with narrow host ranges. It could be argued that much of the information, gained by sacrificing efficiency in detecting new races, is of doubtful practical use.

Dennis (27) has reviewed the taxonomic treatment of physiologic races. In general, they are given numbers or letters which merely indicate the order in which they were discovered. However, Black *et al.* (5) introduced designations for races of *Phytophthora infestans* which are based on numbered major genes for resistance in the host to which each race is virulent. Day (23) has also applied this method to races of *Cladosporium fulvum*. As new resistance genes are identified, any new races that are revealed are assigned corresponding numbers. In the potato, six genes for resistance to *P. infestans* are now recognised (6). Unfortunately, as the number of genes increases, the race designations become long and clumsy. Nevertheless, they are useful to the breeder or pathologist in emphasizing the interdependence of physiologic races and host genes: each is defined in terms of the other. Some of the consequences of this interdependence are discussed below.

The gene-for-gene concept.—Catcheside (18) postulated that resistance or susceptibility is determined by substances produced by corresponding genes in the host and the pathogen. However, the correlation of genes for resistance in the host with genes for pathogenicity in the pathogen was first clearly expressed by Flor (36, 37) in the gene-for-gene-concept arising from his work with the rust (*Melampsora lini*) attacking flax (*Linum usitatissimum*). The concept may be briefly summarized as follows: The ability of a pathogen to produce disease on a host bearing simple major genes for resistance is determined by alleles governing virulence at complementary loci in the pathogen.

The evidence for the gene-for-gene-concept is of two kinds. First, there is the direct demonstration that host resistance determined by a single gene can be overcome by a virulent race of a pathogen which differs from an avirulent race by a single gene. Evidence of this kind is available from Flor's work and from the work of Moseman (90) with *Erysiphe graminis*. Some results from the studies by Shay *et al.* on *Venturia inaequalis* also suggest a gene-for-gene relationship. Virulence to *Malus baccata* var. Dolgo is controlled by a single gene (117, 138). Dolgo is heterozygous for a single major gene for resistance (116). The second kind of evidence depends on the demonstration of the properties inherent in gene-for-gene systems. These properties are revealed by the patterns of physiologic races which are generated by a system of host-resistance genes. These patterns have been discussed by Person (102) in an analysis which shows how the concept may be used tentatively to determine the resistance genotypes of the differential host varieties. Person's method of analysis enabled him to determine not only how many genes were present in each host variety but which genes they had in common.

The work on which Flor based his hypothesis has been criticised. His data on linkage in both flax and flax rust between genes controlling the host-pathogen reactions were criticised by Mayo (87) on the grounds that the tests for linkage were unsatisfactory. Person's (102) criticisms go further than this; they indicate that many of Flor's single-gene differentials had two or more genes for resistance and that the undetected common possession of one gene by two differentials can give the impression of close linkage or even allelism between the other resistance genes. These criticisms do not, however, invalidate the gene-for-gene hypothesis put forward by Flor.

The prediction of host resistance genotypes may be illustrated by Boone & Keitt's (7) studies of the inheritance of avirulence among a group of biotypes of *Venturia inaequalis* on seven apple varieties. The genes for avirulence were identified and separated in recombinant stocks of the pathogen. Each stock had one allele for avirulence. All other loci concerned with reactions on the seven apple varieties were represented by alleles for virulence. From results of inoculations with these stocks it was possible to infer (a) how many genes for resistance were present in each apple variety by observing how many stocks were avirulent on each variety; (b) which genes for resistance were common to two or more varieties by observing avirulence of a single stock on more than one variety. Powers *et al.* (106) tentatively

identified genes for powdery mildew resistance in wheat and barley in the same way.

The gene-for-gene concept thus provides a rational basis for the study of physiological specialisation in several pathogens. In many other plant pathogens, however (including *Puccinia graminis tritici*), the application of the gene-for-gene concept has been hampered by ignorance of the genetics of resistance in the differential host varieties. Person's method of analysis may overcome this difficulty. The work of Finlay (32, 33) on the inheritance of resistance to spotted wilt in the tomato suggests that the concept may apply to some plant viruses. Mode (89) has developed a mathematical model of the evolution of obligate parasites and their hosts based on the gene-for-gene concept. Mode's analysis, like that of Person, reaches the conclusion that an equilibrium between genes for resistance in the host and genes for virulence in the pathogen must be reached if both are to survive.

Griffiths (50) has pointed out that the degree of physiological specialisation a parasite may undergo is determined partly by its ability to propagate itself clonally and partly by the breeding system of the host. If sexual reproduction is obligatory for the production of primary infections, as in the smuts and apple scab, specialised biotypes may not become established unless the host is inbred or vegetatively propagated.

ORIGIN OF NEW RACES

Sexual recombination.—The production of new physiologic races of many plant pathogens may be brought about by sexual recombination. This observation needs little emphasis and has been adequately discussed in other reviews (34, 123, 142). This section merely attempts to bring those discussions up to date.

New races that have arisen in this way have recently been described for several pathogens including *Phytophthora infestans* (120), *Erysiphe graminis* (105), *Ustilago avenae* (51), *Puccinia carthami* (84), *P. graminis tritici* (136), and *P. recondita* (129).

The two mating types of *P. infestans* were recently discovered by Gallegly & Galindo (42, 43) among isolates of the pathogen from Central Mexico. There was no correlation between mating type and host range among the 16 races and some others found in this region (96). More than 100 isolates from North America, Europe, and other parts of the world were of one single mating type when tested with the two from Central Mexico (120). Niederhauser (95) has suggested that the existence of many different races in this area can be attributed to sexual recombination and the occurrence of resistant wild host species. The severe blight epidemics in the Toluca valley of Central Mexico are used to test potato breeding stocks. The results have caused a trend toward the abandonment of breeding for so-called hypersensitivity genes which control resistance to blight and a shift toward breeding for field resistance (54). No hypersensitive genotypes were completely resistant in these extreme conditions. Similar failures of hypersensitive genotypes were found in Northern Ireland by Proudfoot (107).

The irregular distribution of two mating types was also found by Holmes (59) in *Ceratocystis ulmi* (Dutch Elm disease), occurring in Massachusetts. It is likely that some pathogens with no known sexual stage, such as *Cladosporium fulvum*, are still regarded as imperfect because they have been studied in areas where only one mating type is prevalent. If there is a perfect stage it might be expected to occur where the pathogen attacks wild populations of the host as in *P. infestans*.

The contribution of sexual recombination to variation is influenced by other factors. In the heteroecious rusts, sexual recombination takes place on an alternate host. Absence or rarity of the alternate host will prevent or limit sexual recombination. The eradication of *Berberis vulgaris* in North America has probably limited sexual recombination in *Puccinia graminis* (121). However, *B. vulgaris* is still important elsewhere (133).

There is also evidence of physiological specialisation in relation to the alternate host itself. D'Oliviera (29) has presented an example of this in *Puccinia recondita*; he found that the Siberian aecidial host of this species, *Isopyrum fumarioides* (Ranunculaceae), was resistant to Portuguese races of the fungus. LeRoux & Dickson (78) found two groups of biotypes of *P. sorghi* that differed in ability to produce pycnia on two species of *Oxalis*. The sporidia of race 15B of *P. graminis tritici* were reported by Johnson & Green (49, 70) to be avirulent on plants of *Berberis vulgaris*. Sporidia of certain other races produced mixtures of resistant and susceptible type infections on a single plant. These findings suggest that genetic variation in resistance and susceptibility to the sporidia of *P. graminis* might be found between collections of *B. vulgaris*. In autoecious rusts such as *Melampsora lini* (37), genes controlling virulence are expressed in the haplophase. We may postulate that, if two rust haplophases are unable to infect the same host genotype, the chance of crossing between them is likely to be lower than if they were able to.

While sexual recombination has enabled us to determine the genetic control of virulence of several pathogens it is now also used to produce inbred stocks. Dickson *et al.* (28) have used inbred lines of *P. sorghi* in physiological studies in an effort to define the pathogen genotypes they studied and to stabilise their reactions on host plants. The extent of inbreeding is not stated nor is the value of the operation assessed. Another communication (1) states that variation in host reaction is still found after inbreeding *Puccinia sorghi*.

Cherewick (19), in a study of their variability, reported preliminary results of inbreeding cereal smut races. There were differences in variability not only between smuts but between collections of the same species. Cherewick suggested that stable, homozygous smut cultures could be produced by inbreeding and that these would be very useful in testing varieties of cereal crops.

Heterokaryosis and somatic recombination.—The role of these mechanisms in producing variation in fungi has been summarised by Pontecorvo (103, 104) and, in plant pathogens, by Buxton (17). Heterokaryosis provides new phenotypes by the association of unlike nuclei in a single mycelium. It also

provides a store of variation released by changes in nuclear ratio or by the formation of monokaryotic spores or hyphal tips (86). In *Neurospora crassa* the formation of heterokaryons is restricted by heterokaryon-incompatibility factors (45, 46, 58). The transfer of protoplasm between strains that are heterokaryon-incompatible results in cell death (47, 139). Similar incompatibility systems have been described in *Streptomyces* species (10, 11) and may well be found to control heterokaryosis among plant pathogens.

Isolates of wood-rotting basidiomycetes have been included in a single species on the basis of ability to form hyphal anastomoses with each other (72) and by tests of interfertility (97, 111). Fusions between different haploid and dikaryotic mycelia of *Polyporus betulinus* in culture have been described but their genetic significance has not yet been evaluated (14). The production of mutants is associated with heterokaryosis in the agaric *Schizophyllum commune* (108) but it is not known whether this is a common feature of heterokaryosis.

Most pathogens that have the potentiality for heterokaryosis also have the opportunity for somatic recombination. This process depends upon the formation of a diploid nucleus from two haploid nuclei in the somatic mycelium. Recombination of factors in the diploid nucleus takes place by one of two independent processes: mitotic crossing-over and haploidisation. The first recombines linked genes and, in a heterozygous diploid, brings about the homozygosity of loci on the chromosome arm distal to the point of crossing-over. In a heterozygous pathogen, the resulting homozygosity of genes controlling virulence may give rise to new phenotypes. Haploidisation, by contrast, recombines whole chromosomes, balanced combinations surviving, deficient and polysomic nuclei being eliminated. With n chromosomes, 2^n different haploid sets can, in theory, be recovered from a diploid heterozygous for every chromosome. The probable frequencies of these events have been calculated from laboratory studies with *Aspergillus* (104). They are rare but are likely to be important in the absence of a perfect stage.

In a study using induced auxotrophic markers, Buxton (15) has demonstrated that new races of *Fusarium oxysporum* f. sp. *pisi* may be produced in the laboratory as a result of somatic recombination. It is not known whether the recombinants produced from a heterozygous diploid arose by mitotic crossing-over or by haploidisation. Hastie (53) has produced balanced heterokaryons between induced auxotrophic mutants of *Verticillium albo-atrum* from hop. Putative diploid colonies from such a heterokaryon were found to produce strains recombinant for parental markers.

Experiments with similar consequences have been carried out in the rusts, using spore colour and host range as genetic markers. In the earlier experiments by Nelson *et al.* (91 to 93) with *Puccinia graminis tritici*, collections of dikaryotic uredospores of different races were mixed in pairs and inoculated into wheat plants. Heterokaryons were isolated from the mixtures which were recombinant for spore colour or virulence, or both. The initial recombinant cultures had proportions of tri- or quadrinucleate uredospores. In these experiments, recombinants were too infrequent to be explained

merely as associations of a plus mating type nucleus from one parent with a minus mating type nucleus from the other, unless exchange of nuclei were greatly restricted, perhaps by mechanisms like those controlling heterokaryon incompatibility referred to above. Watson (132) and Watson & Luig (134) mixed red-spored race 111 with orange-spored race NR-2. The orange parent was virulent on four host varieties to which the red parent was avirulent; only cultures from red pustules on these four varieties were analysed. Eleven different races were found among a hundred heterokaryons tested. The information on the genetic control of virulence to certain of the host varieties obtained from these somatic studies agrees with similar information obtained by Wilcoxson & Paharia (136) from selfing studies of red race 111 on the alternate host. Some striking dissimilarities between these results and those obtained earlier by Johnson (69) are explained on the grounds that red race 111 is possibly a hybrid between *P. graminis tritici* and *P. graminis secalis*.

Watson & Luig (134) also showed that a new race, produced by mixing two common Australian field races in the greenhouse, was prevalent in the field where it presumably had a similar origin. Watson (132) has noted that reassortment of the four nuclei of two rust dikaryons in compatible pairs would only lead to two non-parental dikaryons, and concluded that somatic recombination must be responsible for the large number of new races found. Similar conclusions were reached by Vakili & Caldwell (130) who mixed red race 2 and yellow race 122 of *P. recondita tritici* and recovered 33 different races, 17 of which were previously unknown. In a later publication Vakili (129) states that some of the phenotypes produced in this and other experiments could have arisen by reassociations of intact parental nuclei. Flor (39) reached similar conclusions in a limited study of vegetative hybridisation in *Melampsora lini*. Vakili further records that no somatic recombinants between the markers "yellow spore" and "virulence on host variety 36" were found. This agrees with the close linkage between these markers established in sexual studies.

New races of *Puccinia graminis tritici* were produced from mixed inoculations by Bridgmon (12) and also from mixtures of *P. graminis tritici* and *P. graminis secalis* by Bridgmon & Wilcoxson (13). Earlier studies (137) had shown that *P. graminis tritici* will anastomose with four other varieties of *P. graminis*.

Somatic recombination has also been observed in the smut fungi. Rowell (109) showed that haploid lines with recombinant mating types were produced by treating with α -radiation a solopathogenic line of *Ustilago maydis*, which was assumed to be diploid. The diploid solopathogenic line was assumed to result from a failure of meiosis during brandspore germination.

The genetical techniques developed for *Neurospora* and yeast were first applied to smuts by Perkins (101) who isolated mutants of *Ustilago maydis* with induced biochemical deficiencies. Holliday (57), using biochemical markers (56), has found that solopathogenic diploids may readily be isolated by plating pieces of immature gall tissue of *U. maydis* on a minimal

medium which will not support growth of parental monokaryons bearing different biochemical markers. The diploids are sporidial and prototrophic and, by means of a replica-plating technique (77), auxotrophic segregants may be readily detected. The incidence of segregation is greatly increased by treatment with ultraviolet light when twin colonies, made up of reciprocal cross-over products, are often found. In contrast to Rowell's (109) findings, the recombinants appeared to be diploid products of somatic crossing-over; no haploids were detected. Markers on three chromosome arms have already been identified and linkage groups established which agree with those found from sexual analysis.

In concluding this section the following speculations seem worth considering. Uredospore colour and virulence markers can be used to produce circumstantial evidence for somatic recombination in rusts. Will a complete somatic analysis, comparable to the promise of this procedure in *U. maydis*, be possible in the rusts? The first step will be to recognise and isolate diploids. This may be difficult since it is not known how stable they may be or what they may look like. If they produce the same spore forms as the dikaryon they may differ in not having a fixed complement of two nuclei per cell. The heterokaryons of *P. graminis* with uredospores having three or four nuclei described by Nelson *et al.* (93), may have been unstable diploids. The inability of the dikaryon of *U. maydis* to grow on a simple minimal medium, thereby allowing automatic selection for haploid or diploid sporidial growth, is a great technical advantage. It prompts one to ask how much effort has been devoted to culturing the haplophase of rusts from sporidia on artificial media.

Once a diploid has been produced, tests must be devised for discriminating between haploid recombinants and diploid recombinants produced by mitotic crossing-over. This discrimination is needed for mapping the markers. In all the rust studies reported to date, somatic recombinants are recognised only as heterokaryons or dikaryons, which is a considerable complication. The autoecious rusts, particularly *Melampsora lini*, offer the most promise because they may produce a greater range of rust genomes, from haploid upwards, on one host species. Also, the same virulence markers can be used in both the haplophase and the dikaryophase.

Adaptation.—In recent years, much time and discussion has been devoted to the distinction between enzymatic adaptation and mutation and selection as agents changing the phenotypes of cultures of microorganisms. Many of the arguments relating to bacteria and yeasts are presented in the papers and discussions of the *Ciba Foundation Symposium on Drug Resistance* (140). The literature on adaptation in fungi was surveyed by Christensen & Daly (21), and on the adaptation of plant pathogen to host by Christensen & DeVay (22) and, more recently, by Buxton (17).

In most attempts to distinguish between these two modes of variation in the virulence of fungal pathogens, technical difficulties have proved insuperable. Buxton (16) has shown that pea root exudates may bring about a change in the virulence of isolates of *Fusarium oxysporum pisi*. The change

takes place *in vitro*, a considerable technical advantage over examples which can only be studied *in vivo*. However, even in this instance, it must still be decided which mechanism is responsible for the change in phenotype. Bradley (9) has provided a striking example of the role of mutation and selection in what appeared, at first sight, to be an example of vegetative hybridisation in an actinomycete. Populations of one strain of *Streptomyces griseus*, exposed for several growth cycles to a sterile culture filtrate of another strain, acquired several genetic characters of the latter strain. Streptomycin resistance was acquired because mutants were selected by a low concentration of streptomycin in the filtrate. A temperate bacteriophage, also in the filtrate, selected resistant mutants. Many of these mutants were also resistant to bacteriophage V₁. Resistance to V₁ was one of the markers supposedly transferred. In this example the individual features of the new phenotype could be analysed independently. Some other aspects of adaptation are discussed in the next section.

Cytoplasmic variation.—The only example of the cytoplasmic control of virulence known to the author was described by Johnson *et al.* (69, 71, 94). These workers found that the progeny of reciprocal crosses between races of *Puccinia graminis tritici*, which were differentiated by the wheat varieties Marquis and Kota, were like their pycnial (i.e., maternal) parents in being either virulent or avirulent on these varieties. The selfing studies of Wilcoxson & Paharia (136) suggested that, for a different race of the fungus, virulence was under nuclear control.

Recent work with saprophytic fungi has shown that the cytoplasm can have important effects on phenotype (48, 66, 115). A first requirement for studies of this kind is a simple method for distinguishing between nuclear and cytoplasmic components of variation. This can be done by means of Jinks's (64, 65) heterokaryon test. The test is made by combining in a heterokaryon two stocks which differ in the character to be investigated. One of these stocks carries a nuclear marker. The heterokaryon, in which both nuclei are present in a common cytoplasm, is then resolved into its component monokaryons which are identified by the marker. If there is no other difference between the recovered monokaryons than that due to the marker, it is concluded that the original heterogeneity was under cytoplasmic control.

By this means, Jinks has shown that spore germination, growth rate, pigmentation, sporulation, perithecia formation, and adaptability to sugars and mercuric chloride in *Aspergillus glaucus* can all be under cytoplasmic control and that these controls respond to selection (64, 65, 67). The capacity of different fungal clones to respond to selection was often under nuclear control while the differences within clones, revealed by selection, was determined by the cytoplasm. In the same laboratory, Arlett (2) has shown that cytoplasmic mutants may be isolated following treatment with acriflavine or ultraviolet light.

In a study of cultural and pathogenic variability in *Septoria avenae*, Hooker (61) concluded that the continued variation shown by serial macrospore isolations was most likely due to heterokaryosis and gene mutation. It

seems likely that some part of the variation described was cytoplasmic in origin like that in *Aspergillus glaucus*. This problem could be resolved by means of the heterokaryon test. This test may also be useful in deciding between enzymic adaptation and mutation and selection as modes of phenotypic change. If the assumption is valid that adaptive enzyme changes only affect the cytoplasm, then, if component monokaryons are recovered from a heterokaryon between an unadapted and an adapted strain, they should be similar in phenotype unless the original difference lay in their nuclei. Such a test could be applied to the adapted forms of *F. oxysporum pisi* obtained by Buxton (16) following treatment with pea root exudates. Similar methods might also be useful for the analysis of strains of *Botrytis cinerea* adapted to concentrations of fungicides which are lethal to wild-type (98 to 100).

Lindberg (81, 82) has described a transmissible disease of *Helminthosporium victoriae* which appears to have some properties in common with the lethal suppressive cytoplasms found by Jinks (68) in aged clones of *Aspergillus glaucus*. Both conditions are transmitted by mycelial contact and bring about pathological changes in a normal mycelium. The disease of *H. victoriae* appears also to be transmitted by extracts which contain no viable fragments of the fungus. Diseased colonies of *H. victoriae* continue to grow slowly while the infected clones of *A. glaucus* die.

Mutation from avirulence to virulence.—The appearance of new physiologic races of phytopathogens has often been attributed to gene mutation. However, spontaneous virulent mutants have rarely been observed under conditions precise enough to allow their frequency to be measured. Watson (131), using four different races of *Puccinia graminis tritici* selected for unusual spore colour or virulence, or both, isolated some spontaneous mutants with increased virulence on the wheat variety Lee. Zimmer & Schafer (143) recovered a virulent mutant of *Puccinia coronata*. Gallegly & Eichenmuller (41) reported the frequent occurrence of mutations to virulence on potato varieties with the resistance gene R_4 in races of *Phytophthora infestans*. These mutants were recovered in cultures from a single zoospore. The cultures were probably monokaryotic originally but became heterokaryotic as a result of mutation during growth.

If avirulence towards a given host genotype is controlled by a single gene in a pathogen then a change in the pathogen to virulence can occur by mutation. Flor (38, 40), Day (25), and Schwinghamer (112, 113) have succeeded in inducing changes of this kind in phytopathogenic fungi. In these experiments, asexual spores or mycelia were treated with a mutagen, x-rays, neutrons or ultraviolet light, and inoculated to resistant hosts which, in most of the examples, were effectively monogenic for resistance. With suitable precautions against contamination, the susceptible lesions produced were attributed to mutation from avirulence to virulence. Day (25) ruled out contamination in his experiments with the tomato pathogen *Cladosporium fulvum* by using an avirulent stock which carried an induced pigment marker character (26). When treated conidia were inoculated directly to the monogenic resistant host, no mutants were recovered. One mutant was re-

covered after a postirradiation period of growth in culture. Reconstruction experiments using known proportions of virulent and avirulent conidia in mixed inoculations showed that a mutation rate of the order of 1.7×10^{-6} was required to recover a single mutant lesion.

Flor (38, 40) selected for his experiments an F_1 hybrid dikaryon between race 1 and race 22 of *Melampsora lini* which was heterozygous for virulence on 16 supposedly monogenic resistant flax varieties. The hybrid was homozygous for avirulence on three other flax varieties. Virulence was recessive and Flor argued that a mutation of a dominant avirulence allele in the heterozygote to the recessive condition would be revealed by the virulence of the resulting homozygote on one of the 16 flax varieties. However, it was pointed out by Day (25) and later also by Flor (40), that a deficiency for a part or even the whole of a chromosome carrying the dominant allele would probably result in a viable and virulent hemizygote. A genetic analysis would be required to see if the mutant in a monokaryon or a homozygous dikaryon was viable and virulent and thus contained only a small deletion or a point change at the locus concerned.

In spite of these restrictions on interpretation, Flor's results are very informative. No mutants were obtained which were virulent on the three varieties to which the F_1 hybrid culture was homozygous for avirulence, or on five of the varieties to which the F_1 hybrid culture was heterozygous for virulence. However, a number of mutants virulent on one or several of the remaining 11 varieties was obtained. A total of three mutants was found on the varieties Birio (two mutants) and Cass (one mutant) in control experiments with the untreated dikaryon. These were also the most common classes of mutants from the x-rayed material.

Person's (102) analysis of Flor's earlier data has revealed that a number of the supposedly monogenic resistant flax varieties apparently have two or more resistance genes, some of which are common to several varieties. It is therefore possible that the rust mutants which attacked a group of varieties did so because these varieties had a common gene for resistance and that a complementary gene for avirulence in the pathogen had mutated. Flor, on the other hand, supposed that there were separate but linked genes for virulence on the varieties of a group and that these were all involved in the same mutational event. Flor's evidence for linkage of such genes is likewise subject to the explanation that different varieties may have a common resistance gene (102).

Schwinghamer (112, 113), in his experiments, used a clone of race 1 of *Melampsora lini* which was known to be heterozygous for virulence at four loci. The locus controlling virulence on the flax variety Dakota was selected for study. Since Person (102) has concluded that the variety Dakota has two resistance genes, we must assume that the rust clone used by Schwinghamer was also homozygous for virulence to the other resistance gene in Dakota. Comparisons were made between the effects of three agents in producing mutants virulent on Dakota. The average maximum frequency of mutants produced by fast neutrons, x-rays and ultraviolet light was 2.0, 1.5, and 0.3

per cent of infections, respectively. Schwinghamer concluded that most of the mutants induced by neutrons and x-rays resulted from chromosome deletions while the ultraviolet light-induced mutants were more likely to have been smaller deletions or intragenic changes.

There is, however, another possible explanation for the occurrence of virulent rust mutants following irradiation of a heterozygous dikaryon, namely, that the virulent mutants were somatic recombinants. Some recent work on *Aspergillus oryzae* and *A. sojae* by Ishitani *et al.* (62, 63) has shown that the rate of occurrence of diploids is enhanced by a factor of 10^4 by treating the conidia of heterokaryons with ultraviolet light. How general this may be in heterokaryons of other fungi is not known; nor is it known whether neutrons and x-rays have a similar effect. However, in irradiated flax rust, it is conceivable that the frequency of diploid nuclei and hence of recombinant diploid nuclei or dikaryons in incipient avirulent infections might be sufficiently high for them to be recovered as virulent lesions on the selective hosts. An admittedly poor test of this theory could be obtained by determining whether the frequency of virulent rust types on a host with several different resistance genes was greater than would be expected by multiple mutation. Flor himself found that two of 94 mutants produced from x-ray treatments were of this kind in that they each attacked two different varieties or variety groups although they had only been selected for virulence on one.

As Flor (36) has pointed out, mutation experiments may ultimately be used to assess the relative merits of different host resistance genes. These could be compared in terms of the relative mutability of their complementary pathogen avirulence genes. Thus, if few or no mutations to virulence were recovered on one resistant host it would be assumed that this genotype would be likely to remain resistant for a longer period than others which were more frequently infected in the test. The argument assumes that a gene with a low induced mutation rate also has a low spontaneous mutation rate. It is worth pointing out that any such experiments would have to be rigorously controlled to prevent the escape of pathogens with new virulence genes which could render years of breeding work valueless.

The basis of physiologic specialisation.—The high degree of specificity that exists between pathogen and host in those associations where there are clearly defined physiologic races is a challenging problem. It has been approached in several ways.

Serological methods have been used in an attempt to develop an *in vitro* test to distinguish between races. Although Tempel (125, 126) has distinguished different species and *formae speciales* by this means, it is of doubtful value for distinguishing physiologic races. He has reviewed the literature (127) on serological investigations in fungi causing plant diseases. He also presents the detailed results of his own investigations with *formae speciales* of *Fusarium oxysporum*. Tempel concludes that culture liquid and mycelial extracts contain various autolytic products of mycelium glycoproteins which include polysaccharides and proteins. While the polysaccharides are much more constant and stable than the proteins, antibodies to them are only pro-

duced by immunization with microconidia. Douglas & Garrard (30) failed to separate strains of *Actinomyces scabies* into pathogenic and non-pathogenic forms by serological tests. McKee (85) prepared an antiserum by intravenous injection of zoospores of race 1,4 of *Phytophthora infestans* into rabbits. The antiserum immobilised zoospores at a dilution of 1:1000 and gave a sharp zone of precipitation on gel diffusion plates. However, eight other races showed few differences in sensitivity and gave identical precipitation zones.

The correlation of cultural features with pathogenicity or virulence has also been examined. Hodgson (55) compared the growth of single isolates of the four races 0; 1; 1,2; and 1,2,3 of *Phytophthora infestans* on a synthetic medium. There were significant differences between the races in response to supplementing the medium with various growth-promoting substances. Hodgson concluded that these differences could only be considered important in distinguishing races if it could be shown that different isolates of a single race were uniform in this respect. Experiments by Thurston *et al.* (128) showed that six isolates of race 0 from the same potato field showed a greater variation in growth on unsupplemented synthetic media than the variation found among single isolates of the five races: 1; 1,4; 4; 2,4; and 1,2,4. These authors concluded that many isolates of a given race should be studied before differences in ability to use certain chemicals can be accepted as racial characters.

A source of error in this type of experiment has been pointed out by Taber (124) who found that quite small differences in inoculum size have disproportionate effects on final mycelium dry weights in liquid culture studies. The minimal effective inoculum size was shown to vary with different media in Hall's (52) studies of *P. infestans* on artificial media.

Lewis (80) put forward a nutrition hypothesis to account for physiological specialisation and suggested that although nutritional differences may not be demonstrated *in vitro* they may occur *in vivo*.

Induced biochemical mutants of several plant pathogens have been studied to see if there are changes in virulence which can be correlated with the mutant requirements. Much of this work has been reviewed already by Keitt & Boone (73), Keitt *et al.* (74), and by Schwinghamer (114). It may be noted in passing that in some saprophytic fungi the difficulties of producing auxotrophic markers have been overcome by various techniques. Lester & Gross (79) claimed to have obtained yields of auxotrophic mutants as high as 90 per cent by irradiating an inositol-requiring strain of *Neurospora crassa*. The results from studies of auxotrophic mutants in *Venturia inaequalis* (8, 76), *Cladosporium fulvum* (24), and *Colletotrichum lagenarium* (31) have been disappointing. Certain mutants were non-pathogenic but were non-pathogenic on all the host varieties tested. Some non-pathogenic auxotrophic mutants of *V. inaequalis* had their pathogenicity restored when inoculated host leaves were supplemented with the mutant requirements while others remained non-pathogenic. The changes reported affected pathogenicity rather than virulence.

However, from similar studies with the plant pathogenic bacterium *Erwinia aroideae*, Garber (44) has developed a nutrition-inhibition hypothe-

sis. Two sets of circumstances in the host favour pathogen virulence; adequate nutrition and non-inhibition. If either or both are unfavourable the result is avirulence.

A most promising line of investigation would now seem to be the isolation of many virulent mutants in a haploid phytopathogenic fungus which can be cultured on a simple synthetic medium. A detailed comparison of these mutants with the avirulent wild-type should reveal how their metabolism has been altered so that they avoid triggering the mechanism for host resistance, be it nutritional or inhibitory. The genetic analysis of such changes could then be attempted by some of the techniques discussed in this review.

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MICROBIAL NUTRITION

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Experimental nutrition has engaged the attention of microbiologists for a long time. Inasmuch as the very foundation of microbiology rests on the ability to grow microorganisms in pure culture, the nutritional requirements of bacteria, yeasts, and molds became early a practical problem. The understanding of the basic nutritional requisites for the growth of microorganisms has led to a gradual replacement of complex media by chemically defined media adequate for luxuriant growth of many species. Such media have proved to be an asset in studies of bacterial function.

The identification of nutritional factors required for microbial growth, which occupied so much of the time and energy of microbial nutritionists had, as an extra bonus, the discovery of new factors some of which were subsequently identified as vitamins for other species, including man. While such studies continue to occupy the attention of microbiologists, the emphasis in nutrition in recent years has been directed towards an understanding of the functions of the nutrients required for growth. In addition, since it is manifest that the nutritive must penetrate the cell and subsequently be used properly by the biochemical machinery of that cell, studies on permeability and the metabolic fate of nutrients are quite abundant. Inasmuch as certain enzyme systems appear to reside in subcellular particles, future attention must be focussed upon elucidation of the mechanism of internal permeation of nutrients. Finally, it is becoming increasingly apparent that antagonisms and interactions among nutrients as well as the character of the physical environment influence markedly the nutritional demands of an organism. Unless strict attention is given to such factors as the age of the culture, the pH, temperature of incubation, oxidation-reduction potential, etc., variable results are obtained and disagreement among workers in the field is reported.

The present review covers only portions of the work appearing in the literature during the past 18 months and touches on some of the problems mentioned.

INFLUENCE OF NUTRITION ON FORMATION OF ENZYMES

It is important to emphasize the fact that the adequacy of nutrition for a given organism cannot be based entirely on how well that organism grows, but rather on how well it functions. Equal growth does not necessarily imply equal function. A bacterial cell does not possess at any one time all of the enzymes required for dealing with all possible environments. The actual enzymic constitution of the microbial cell is determined to a large extent by the external conditions obtaining during the formation of the individual cell.

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To put it another way, the actual enzymic constitution of the cell is that portion of its genetic potential that is selected by the environment during the formation of the cell. Thus, genetic expression is affected by the environment, and the presence of a particular gene does not necessarily mean that the corresponding character will be expressed. Of the many environmental agents operative, one of the most important and widely studied is that of nutrition.

The nutritional requirements for the formation of arginine decarboxylase by *Escherichia coli* have been studied by Melnykovych & Snell (1). Albeit the organism is capable of synthesizing a supply of amino acids sufficient to permit good growth, supplementary amino acids must be provided to allow optimal synthesis of arginine decarboxylase. In non-aerated cultures, arginine, methionine, tyrosine, and asparagine replaced in large measure the effects of a casein digest. The presence of iron was also stimulatory. When aerated cultures were studied (where growth was much heavier) cells with high decarboxylase activity were produced when glutamic acid and iron were supplied in addition to these amino acids. The function of iron in this system is not yet clear. These observations re-emphasize the importance of the nutritional environment in determining the biochemical potentiality of the harvested cells. Billen & Lichstein (2) earlier observed a similar relationship between the nutritional requirements for growth and hydrogenase synthesis in *E. coli*. Perhaps a type of physiological control mechanism exists in such cases whereby relatively unimportant or "luxury" enzymes are not produced until the full complement of essential enzymes are synthesized. However, in the presence of excess amino acids these building blocks can be used to express more fully the genetic potential of the organism.

Jayko & Lichstein (3) reported on the nutritional factors concerned with lecithinase (α -toxin) production by *Clostridium perfringens*. In agreement with other workers, they found that the addition of enzymatically hydrolyzed casein to a synthetic medium in place of the constituent amino acids supported lecithinase synthesis, while acid-hydrolyzed casein was only partially active under these conditions. Synthetic peptides, notably glycyl-L-asparagine, stimulated both growth and lecithinase synthesis when incorporated into the chemically defined medium. Of interest was the finding that the amino acid concentration for optimal growth was higher than that needed for optimum lecithinase production, and increases in amino acid concentration appeared to mask the stimulatory effect of the peptides on lecithinase synthesis. Alanyl-DL-asparagine and glycyl-D-asparagine failed as substitutes for glycyl-L-asparagine, and the D form of glycylasparagine in the presence of its L counterpart caused an inhibition of lecithinase biosynthesis. A surprisingly high degree of peptide specificity is indicated by these results.

Shah & King (4) demonstrated that resting cell suspensions of *Bacillus subtilis* harvested from a simple synthetic medium possessed relatively low reductive amination activity, while similar suspensions derived from nutrient broth, casein hydrolyzate, or a mixture of amino acids simulating casein hydrolyzates exhibited good enzyme activity. The addition of a variety of

amino acids either singly or in simple mixtures yielded bacterial cells with activities up to about half of that attained with complex media. However, no single amino acid exhibited significant stimulation of reductive amination activity. It was concluded that a high enzyme yield requires a medium with a complex amino acid relationship, and that the performance of such media cannot be explained directly in terms of a few active components.

Several papers have appeared on the nutritional requirements for proteinase biosynthesis by bacteria. Rabin & Zimmerman (5) investigated this enzyme in *Streptococcus liquefaciens*. Proteinase activity of cell suspensions was measured by assaying the amount of tyrosine solubilized when a casein substrate was used. The basal medium contained casein, lactose, purines, pyrimidines, vitamins, and salts. It was shown that proteinase activity can occur in a medium devoid of protein by substituting 12 essential amino acids for the casein. These included glutamate, valine, histidine, serine, methionine, leucine, isoleucine, arginine, cystine, lysine, tryptophan, and threonine. Of interest was the observation that high concentrations of arginine were needed for good proteinase synthesis. Also, the presence of a fermentable carbohydrate such as glucose, galactose, or lactose was essential for enzyme synthesis. In a subsequent paper the purine, pyrimidine, and vitamin requirements for proteinase biosynthesis by *S. liquefaciens* were reported [Hartman, Zimmerman & Rabin (6)]. Adenine and uracil best satisfied the purine and pyrimidine requirement while pyridoxal and riboflavin were the only vitamins demonstrated to be required by the washed cell suspensions. More recently, Georgi (7) studied the proteinase derived from *Micrococcus lysodeikticus*. Proteolytic activity of the extracellular enzyme was determined by following the change in viscosity of gelatin in an Ostwald viscosimeter. Complex media were employed but proteinase activity did not necessarily parallel growth. For example, peptone as a nitrogen source supported somewhat less growth than the Pasteur Institute meat broth, and proteolytic activity was present in the former medium but not in the latter. Dialysis of the meat broth yielded a proteinase which was increased markedly by the addition of calcium chloride.

Gibbons & Doetsch (8) isolated an obligative anaerobic, ureolytic organism from the bovine rumen and identified it as related to *Lactobacillus bifidus*. Urease production by this organism was impaired by the presence of high concentrations of certain nitrogenous substances and by urea, $(\text{NH}_4)_2\text{SO}_4$, and Mn. It was observed that the inhibitory effect of Mn was overcome by the presence of iron.

The influence of nutrition on the synthesis of penicillinase by *Staphylococcus aureus* was investigated by Kaminski *et al.* (9). Of the amino acids necessary for optimal growth, glutamate and leucine stimulated penicillinase production while arginine and glycine decreased significantly the synthesis of the enzyme. Of the amino acids not required for growth, tyrosine stimulated penicillinase synthesis, whereas isoleucine, methionine, threonine, and serine were inhibitory. The effect of carbon source in the medium revealed that maltose raised the level of penicillinase about threefold compared to that

observed with glucose. The only other carbon source which showed a significant degree of stimulation was mannitol, while starch was definitely inhibitory. Of interest was the finding that isoleucine failed to exert an inhibitory effect on penicillinase synthesis in the presence of maltose. The incorporation of the purines, adenine, guanine, or xanthine resulted in definite inhibition of enzyme synthesis, but the pyrimidines, cytosine or uracil were without effect.

The production of amylolytic enzymes by *Aspergillus niger*, *B. subtilis*, and *Bacillus polymyxa* was reported by Dunn *et al.* (10). Perhaps of most interest was the finding that the incorporation of phytic acid or calcium phytate into synthetic media markedly enhanced amylase activity.

INFLUENCE OF NUTRITION ON FORMATION OF PRODUCTS

The biosynthetic pathways of a microorganism are influenced by the nutritional environment which may permit or preclude the development of metabolic patterns with which the cell is genetically endowed. Studies directed towards the development of growth media suitable for the production of antibiotics, vitamins, and other components in high yield have been abundant. In addition to the fundamental aspects of such investigations the increased yields of certain products have had industrial significance.

A chemically-defined medium for the production of synnematin by *Cephalosporium salmosynnematum* and *Emericellopsis terricola* var. *glabra* has been described by Bhuyan & Johnson (11). The maximum rate of antibiotic production was obtained under conditions which supported a very slow rate of growth. The medium consisted of glucose, ammonium sulfate, calcium carbonate, and salts, plus biotin for *E. terricola*. This simple medium permitted a rapid growth rate during the growth phase and a slow growth rate during the antibiotic synthesis stage. The slow rate of growth during the period of synnematin production was obtained by feeding glucose intermittently (at 12-hr. intervals) to the fermentation after the end of the growth phase. A simple defined medium has been formulated which permits growth of *Streptomyces aureofaciens* from a spore inoculum with the synthesis of 7-chlorotetracycline [McCormick *et al.* (12)]. The medium contained glycerol as the sole carbon source and ammonium salts as the sole nitrogen source plus other inorganic salts. Supplementation of this medium with other defined ingredients improved the yield of antibiotic. An interesting report by Prokofieva-Belgovskaya & Popova (13) revealed that the poor quality of certain batches of corn steep liquor used for the production of chlorotetracycline by *S. aureofaciens* results largely from their high content of inorganic phosphorus. The utilization of amino acids during growth and production of an antifungal antibiotic by *B. subtilis* was studied by Majumdar & Bose (14).

A comprehensive investigation of nutritional factors concerned with riboflavin biosynthesis by the yeast *Candida flaveri* was made by Goodwin & McEvoy (15). Growth and flavinogenesis were maximal after 2 to 3 days incubation in a chemically defined medium containing $(\text{NH}_4)_2\text{SO}_4$ as the sole source of nitrogen. The $(\text{NH}_4)_2\text{SO}_4$ concentration required for maximal flavinogenesis was about twice that needed for optimal growth. The synthesis

of riboflavin was inhibited by traces of iron and reduced by many amino acids which were stimulatory for growth. On the other hand, glycine, serine, ornithine, arginine, and urea stimulated both growth and flavinogenesis. Of the purines and pyrimidines tested, only xanthine and, to a lesser degree, guanine and uric acid stimulated riboflavin formation. Clapper & Meade (16) presented data demonstrating that the riboflavin content of growing cells of *Streptococcus faecalis* increased as the folic acid concentration of the medium was increased. They showed also that resting cell suspensions synthesized riboflavin in proportion to the folic acid available. The mechanism of this interesting vitamin interrelationship was not elucidated.

The influence of nutrition on bacterial pigment formation has been investigated by DeMoss & Happel (17). They reported that under certain conditions violacein production by *Chromobacterium violaceum* was a linear function of the vitamin B₁₂ concentration in the medium. In a subsequent report [DeMoss & Evans (18)], the biosynthesis of violacein was studied in non-proliferating cells. In this case, L-tryptophan was the sole carbon source required for pigment synthesis. The production of pyocyanin by washed cell suspensions of *Pseudomonas aeruginosa* was investigated by Frank & DeMoss (19). Synthesis of this phenazine pigment required magnesium, phosphate, a source of sulfur, and an amino acid substrate. The results of isotope experiments revealed that essentially all of the pigment was formed from the added amino acid substrate. The inability to dissociate pyocyanin synthesis from growth makes difficult the decision as to which of the substrates tested may be specific precursors of pyocyanin. During studies on the enhancement of pigment production of *P. aeruginosa* by *Serratia marcescens*, Chmura & Pelczar (20) found that the incorporation of proline into the medium increased pigment synthesis markedly.

The *in vitro* production of a lethal toxin by a virulent strain of *Bacillus anthracis* growing in continuous culture in a mixture of serum plus a tryptic digest of meat, was reported by Harris-Smith, Smith & Keppie (21). Nutritional studies revealed the importance of large molecular components of serum in toxin production. The authors suggest that the serum may provide large molecular intermediates essential for toxin synthesis or the serum may protect the toxin against the action of a toxin-destroying system by acting as competitive enzyme substrates. Of considerable importance is the fact that this toxin-destroying system appears to account for the failure of previous workers to find the toxin *in vitro* while its *in vivo* formation has been demonstrated abundantly. Puziss & Wright (22) studied the carbohydrate metabolism of *B. anthracis* in relation to the elaboration of protective antigen. It was found that the antigen is produced during growth only in the presence of a readily utilizable source of carbohydrate. Of those tested, only glucose, sucrose, maltose, fructose, dextrin, and glycerol supported significant production of the antigen. The requirement for bicarbonate in the elaboration of antigen demonstrated originally by Gladstone (23), was confirmed and extended.

A method has been developed for the study of tetanus toxin formation

within the bacterial cell [Miller, Eaton & Gray (24)]. The influence of several factors on the synthesis of intracellular toxin was studied, one of which was that of the character of the medium. An increase in internal toxin of approximately 100-fold per cell was noted when pancreatic digest toxin media or defined toxin media containing glycyl-L-histidine were employed. The importance of the nutritional background in the synthesis of toxin by *Clostridium tetani* was demonstrated by the earlier work of Mueller & Miller (25, 26).

During studies on the relationship between dipicolinic acid and heat resistance of bacterial endospores, Church & Halvorson (27) inspected the effect of nutrition on the dipicolinic acid content of spores of *B. cereus* var. *terminalis*. The level of yeast extract in the sporulation medium was important since reduction of this component lowered the dipicolinic acid content from 74 to less than 0.2 $\mu\text{g.}/\text{mg.}$ spores. The level of dipicolinic acid was relatively unaffected by the presence of various amino acids except for phenylalanine, in which case it was reduced to about 0.6 $\mu\text{g.}/\text{mg.}$ spores. The addition of several B vitamins was found to increase the content of dipicolinic acid when the level of yeast extract was reduced.

Chao & Foster (28) described the isolation of a spore-forming rod belonging to the *Bacillus megaterium*-*B. cereus* intermediates. The distinctive property of the organism was its ability to produce exceptional quantities of L-glutamic acid as the chief product of metabolism (up to 12.5 mg./ml. medium). The most interesting observation was the regulatory role of biotin. Glutamate synthesis took place at the expense of cell synthesis when the concentration of biotin was limiting, while cell production occurred at the expense of amino acid synthesis in non-limiting concentrations of the vitamin. The mechanism of this regulation was not elucidated.

The formation and accumulation of large amounts of S-adenosylmethionine by *Candida utilis* when grown in culture media supplemented with L-methionine was reported by Svihla & Schlenk (29). Localization and storage of S-adenosylmethionine in the vacuole was demonstrated by ultraviolet microscopy.

The synthesis of flagella by amino acid-requiring mutants of *Salmonella typhimurium* was investigated by Kerridge (30). The advantages of studying flagella include the fact that they consist only of protein (flagellin) and this protein can be isolated and purified quite easily. The disadvantage is the fact that the quantitative estimation of flagellar synthesis is difficult. Flagella regeneration was examined when the organisms were incubated with a source of nitrogen and energy but in the absence of the amino acid required for growth. The results could be grouped as follows: (a) mutants requiring for growth an amino acid which is also present in flagellin, and unable to regenerate flagella in the absence of this amino acid; (b) mutants requiring for growth an amino acid not present in flagellin, and able to produce flagella in the absence of this amino acid; and (c) mutants requiring for growth an amino acid present only in small amount in flagellin and able to regenerate flagella in the absence of this amino acid.

INFLUENCE OF TEMPERATURE OF INCUBATION ON THE NUTRITIONAL REQUIREMENTS OF MICROORGANISMS

That the physical environment influences the nutritional demands of microorganisms has been well documented. Such factors as temperature of incubation, oxidation-reduction potential, and hydrogen ion concentration must be carefully controlled in any precise study of the nutritional requirements of a given organism. For example, changes in the temperature of incubation may alter the nutritional demands of microorganisms and, in most instances, where this phenomenon has been demonstrated, an increase in temperature is accompanied by a gain in nutritional requirement. In the past few years considerable attention has been devoted to this aspect.

During studies on the nutrition of *Saccharomyces cerevisiae*, Begue & Lichstein (31) observed that several strains were unable to initiate growth or grew very poorly at 38°C. in a chemically defined medium adequate for optimum growth at 30°C. In contrast, these organisms proliferated equally well at both temperatures after inoculation into a complex medium. Subsequent investigation revealed that the active component of the complex medium was replaceable by calcium pantothenate. Addition of this vitamin to the defined medium supported growth of the yeasts at 38°C. to the extent of 40 to 82 per cent of that obtained at 30°C. in the same medium containing β -alanine instead of pantothenate. It appears that the synthesis of pantothenic acid accomplished by these organisms at 30°C. is in some manner prevented by incubation at the elevated temperature. These results are reminiscent of those reported by Maas & Davis (32) who obtained a mutant strain of *E. coli* which required added pantothenate for growth initiation only when the temperature of incubation exceeded 30°C. They concluded, on the basis of further experimental study, that the mutation resulted in the production of an altered excessively heat-labile enzyme catalyzing the biosynthesis of pantothenic acid.

Of interest in this connection are the detailed studies of Sherman (33) on the effects of elevated temperatures on the growth and inheritance of *S. cerevisiae*. He observed a decreased ability of the yeast to proliferate at 40°C. However, growth at the elevated temperature did occur when the concentration of yeast extract in the medium was raised from 0.5 to 2 per cent. The inclusion of oleic acid (1 μ mole/ml.) also promoted growth of the yeast at 40°C. when added to media containing 1 per cent yeast extract but not 0.5 per cent yeast extract. It is possible, in view of the findings of Begue & Lichstein (31), that the yeast extract was supplying pantothenic acid for growth at the elevated temperature, or that multiple nutritional requirements were induced by the high temperature and that these were satisfied by the yeast extract. Also, since oleic acid exhibits surface active properties it is possible that its role may be attributable to enhancement of penetration of some required nutrient into the cell as well as serving as a nutritive itself. On the basis of genetic studies, it was concluded that the elevated temperatures induced respiratory-deficient mutants.

Bird & Gots (34) isolated a strain of *E. coli* which differed from the usual wild-type in that it did not initiate growth in a synthetic salts-glucose medium. Addition to this medium of either methionine or *para*-aminobenzoic acid (PAB) resulted in growth. However, the requirement for methionine or PAB was manifest only when incubation was carried out at 37°C. At room temperature (21 to 24°C.) good growth was obtained in the minimal media, but the addition of homocysteine imposed a requirement for methionine or PAB. The authors concluded that the temperature-sensitive growth requirement appears to be the result of a deleterious reaction involving homocysteine as substrate, which interferes with the function of PAB in the biosynthesis of methionine. Since homocysteine was not inhibitory to wild-type *E. coli*, the authors suggest that this strain may catalyze a reaction diverting homocysteine from its role as a methionine precursor to the production of an inhibitor. This reaction would presumably be absent in wild-type *E. coli* or under suppressive control by some regulatory system.

Studies on the nutritional requirements of *Pasteurella pestis* at 27° and 37°C. were made by Higuchi & Carlin (35). At the lower temperature excellent growth was supported by a medium containing D-xylose, a mixture of several organic and inorganic salts and 10 amino acids. However, the nutritional requirements of this organism were more exacting at 37°C. than at 27°C., which is in agreement with the earlier results of Hills & Spurr (36). The minimal medium adequate for growth at 27°C. was supplemented with DL-isoleucine, thiamine, pantothenate, biotin, and a marked increase in the concentration of MgSO₄. The inclusion of isoleucine which had no effect on growth at 27°C. was essential for growth at 37°C. The addition of the B vitamins, while not demonstrated to be absolute requirements, appeared to promote better growth. The MgSO₄ was definitely stimulatory. Of interest was the finding that the four avirulent strains tested initiated growth rapidly at 37°C. in the modified medium, while the seven virulent strains exhibited a pronounced lag. This difference was not apparent at 27°C. In a subsequent paper (37), it was found that the addition of calcium ions in a concentration of 0.002-0.004 M permitted rapid growth of the virulent strains at 37°C. while having no effect on the growth of the avirulent strains studied. Furthermore, there appeared to be some relationship between virulence and the requirement for calcium ions. For example, the loss in virulence after continued transfer in aerated liquid media at 37°C. was prevented by the presence of calcium salts. The authors suggest the possibility of the development of a selective plating medium for the differentiation of virulent and avirulent strains of *P. pestis* based on the calcium requirement.

The growth of obligate thermophiles as a function of the cultural conditions employed was reported by Long & Williams (38), who employed seven strains of *Bacillus stearothermophilus*. Of the several complex media studied, only tryptose basamin glucose broth permitted growth of six of the seven strains at 37° as well as 55°C. It appears probable that the nutritional requirements for growth at 37°C. exceed those needed for cell proliferation at 55°C. This had been reported earlier by Campbell (39) who emphasized the increased nutritional demands at decreased temperature of incubation.

Hertman & Ben-Gurion (40) studied pesticin formation by *P. pestis*. Pesticin is a bacteriocin-like material whose formation can be induced by ultraviolet irradiation. Nutritional studies resulted in the development of a defined amino acid medium which would support pesticin biosynthesis. The relationship between temperature of incubation and the amino acid requirements for pesticin production revealed that leucine and isoleucine were required at 37°C., while at 27°C. deletion of these amino acids was without effect.

PEPTIDES

An interesting but troublesome problem associated with microbial nutrition is that the requirement for a particular nutrient may not be constant but rather may vary depending in part on the nature and concentration of other chemical components in the medium. This type of relationship is demonstrated very nicely by the reports of peptide requirements resulting from amino acid imbalances. The recent report by Demain & Hendlin (41) has been most instructive in this regard. They studied the growth stimulation of a mutant strain of *B. subtilis*, obtained by ultraviolet irradiation, by glycine peptides. It was observed that the inclusion in the medium of a tripeptide of glycine decreased markedly the length of the lag phase and increased the exponential rate of growth. Of particular importance was the finding that the peptide was not required when the amino acids of the medium were deleted. The inhibitory amino acid was found to be histidine. Apparently then, the peptide was required only under conditions of amino acid imbalance. The locus of histidine inhibition was not elucidated. The authors suggest that one should anticipate peptide requirements to be found more often among the exacting microorganisms whose complex requirements necessitate the inclusion of amino acids into the medium.

Another type of peptide-amino acid relationship is seen by the work of O'Barr, Levin & Reynolds (42). They investigated the interrelationships of amino acids in the nutrition of *Leuconostoc mesenteroides*. Five different amino acid antagonisms on the growth of this organism were noted. These included the amino acid pairs: L-threonine-L-serine; L-serine-glycine; L-threonine-glycine; L-alanine-glycine; and L-alanine-L-serine. In all instances, peptides containing one of the antagonistic pairs was more effective than the free amino acid in relieving these antagonisms. For example, glycyl-DL-serine was more effective than L-serine, while glycyl-DL-threonine was more active than L-threonine in this regard. The authors suggest that in these relationships the free amino acid is antagonized during the absorption process while the peptide penetrates without inhibition. Shiota, Folk & Tietze (43) investigated the competitive inhibition of lysine utilization in *Lactobacillus arabinosus* and *L. mesenteroides* by S-(β -aminoethyl) cysteine (AEC). They established that AEC is an effective competitive inhibitor of lysine for *L. mesenteroides* and, to a lesser degree, for *L. arabinosus*. Perhaps of most interest was the finding that certain lysine peptides were more active than lysine itself in reversing the inhibition caused by AEC, and furthermore the dipeptides tested (L-lysylglycine and glycyl-L-lysine) reversed the inhibition in a

competitive manner. It was conjectured that the dipeptides are not hydrolyzed to free amino acids but are utilized as such or as lysine derivatives.

During studies on the nutritional factors concerned with growth and lecithinase production by *C. perfringens*, certain interrelationships of amino acids and peptides were found [Jayko & Lichstein (3)]. Increasing the concentration of amino acids in the synthetic medium two- to fivefold resulted in improvement of growth, while increasing the concentration to sixfold resulted in slight inhibition of growth. Little or no effect on lecithinase titers was found under these conditions. Of particular importance was the finding that the stimulatory effect of certain simple peptides on lecithinase production was reversed completely when these peptides were added to the synthetic medium fortified with a fivefold increase in concentration of amino acids. It appears that amino acid toxicity must be considered in toxin synthesis.

Turning now to other aspects of the nutritional role of peptides, a provocative article on the specificity of peptides was published by Woolley & Merrifield (44). Data on several peptides possessing vitamin and hormone activity were presented with the suggestion that the specificity among biologically active peptides appears not to be as exacting as might have been expected from experience with the water-soluble vitamins. Perhaps of most interest to the microbiologist are the data on strepogenin. In recent years several pure peptides exhibiting strepogenin activity have been isolated and identified. One such peptide isolated from partial hydrolysates of insulin is serylhistidylleucylvalylglutamic acid (SHLVG). Another peptide isolated from digests of ribonuclease and possessing the same five amino acids was identified as valylhistidylglutamylserylleucine (VHGSL). When tested for strepogenin activity in *Lactobacillus casei*, VHGSL was 2.3 times as potent as SHLVG. On the basis of such results, the authors suggest that the amino acid sequence in the two pentapeptides containing the same amino acids cannot be the crucial factor determining their growth-promoting activity. Further work with other peptides emphasizes the fact that a variety of peptides exhibit strepogenin activity and, moreover, there is little structural resemblance among those which have this activity. There is apparently some specificity, but neither the sequence of amino acids nor the exact nature of the amino acids appears to be critical for biological activity.

It would be appropriate before leaving the subject of strepogenin to mention the work of Kihara & Snell (45). Employing growth of *L. casei* as a parameter, they found that crude digests of casein were superior to digests of purified casein prepared with recrystallized trypsin. Much of the difference in response to the two digests was found to be caused by non-peptidic substances present in the crude digest. Furthermore, spermine and spermidine were shown to possess pronounced growth-stimulating effects for *L. casei*. The authors point out that since spermine and spermidine are widely distributed in nature, it is probable that previous assays for strepogenin in crude materials have included the growth response to these polyamines with that attributed to peptides. Furthermore, it is perhaps understandable on this basis that none of the purified peptides reported to possess strepogenin activity promote growth to the same extent as that observed with crude

preparations. It is hoped that an elucidation of streptogenin will come shortly from one of these two laboratories.

Leach & Snell (46) have made an important contribution to our understanding of the nutritional role of peptides. Studies with *L. casei* revealed that certain glycine peptides are accumulated via a different pathway from that of free glycine. Furthermore, the glycine peptides are accumulated at a faster rate than glycine and appear to be hydrolyzed to the free amino acids prior to incorporation into protein. The peptide used was L-alanyl-glycine-2- C^{14} . The pertinent finding was the lack of competition of unlabeled glycine during the accumulation of radioactivity from the labeled peptide.

Smith & Higuchi (47) reported on the utilization of peptides during growth of *P. pestis*. In general, growth was not enhanced by supplying amino acids in peptide form except in the case of diglycine or triglycine peptides of glycine.

Certain nutritional relationships in *L. arabinosus* were studied by Ifland *et al.* (48), employing the inducible malic enzyme as a parameter. It was found that peptides of phenylalanine as well as phenylpyruvic acid replaced phenylalanine in stimulating the synthesis of malic enzyme by washed cell suspensions. On the basis of their results, it was suggested that peptide, keto acid, and amino acid are converted by separate routes to an active form of the amino acid required for enzyme biosynthesis.

Straka & Stokes (49) demonstrated that cells of several species of the genus *Pseudomonas* and *E. coli* manifest increased nutritional requirements after quick freezing and subsequent storage at temperatures between -7° and -29°C . On the basis of rather limited nutritional study the authors suggested the possibility that peptides may be required by such cold injured cells.

VITAMINS

Vitamin B₆.—Holden (50) has studied the degradation of intracellular nucleic acid and leakage of fragments by *L. arabinosus* grown under conditions of vitamin B₆ sufficiency and deficiency. Cells harvested from a complete medium during the early stages of exponential growth were found to contain two to three times as much nucleic acid as similar cultures near the end of active growth. During incubation of cells, harvested from the complete medium in phosphate buffer (pH 6.5) at 37°C ., a degradation of nucleic acid occurred which was most pronounced in early exponential phase cells and almost absent in cells nearing the end of active growth. Leakage of large quantities of material into the buffer took place only from logarithmic phase cells incubated in the absence of glucose. When glucose was present, leakage was slight regardless of the growth phase, and degradation itself was reduced and actually demonstrable only in cells from the early logarithmic phase. Analysis of the cell extracts for RNA and DNA revealed that the loss of 260 $m\mu$ absorbing material from the hot HClO_4 extracts occurred entirely at the expense of RNA. Vitamin B₆-deficient cells, in contrast, degraded nucleic acid almost equally in the presence or absence of glucose and did not retain intracellularly the products of this degradation when incubated in the pres-

ence of glucose. The author suggests the similarity between his results on RNA degradation with those obtained when chloramphenicol-treated bacteria are incubated in antibiotic-free media. It would be interesting to know the relationship if any between this phenomenon and the observations made by Gale (51) and by Lichstein & Umbreit (52) which were made with Gram-negative bacilli and called decay and aging, respectively.

Another interesting paper from the same laboratory deals with the effect of vitamin B₆ deficiency on glutamic acid accumulation by cells of *L. arabinosus* [Holden (53)]. The general characteristics of glutamate accumulation in this organism were described in an earlier paper (54). The pertinent findings were that a vitamin B₆ deficiency reduced the amount of glutamate accumulated by cells of *L. arabinosus*, but did not reduce the initial rate of accumulation. Furthermore, the accumulation ability of resting cells of vitamin B₆-deficient *L. arabinosus* was not improved by preincubation with pyridoxal or pyridoxal phosphate. These results and others suggest that the role of vitamin B₆ in this system is of an indirect nature. The apparent contradiction in findings between the bacterial system and that proposed from studies with mammalian cells obviously requires further investigation. In a subsequent short communication, Holden (55) presented new and revealing information. The early reduction in glutamate accumulation noted with vitamin B₆-deficient cells was almost entirely eliminated when acetate, NH₄⁺, and vitamin B₆ were added to the uptake buffer. Vitamin B₆ alone was inactive, acetate and NH₄⁺ individually had very slight effect while a combination of the latter two substances showed 40 to 70 per cent of the effect of the complete mixture. Perhaps of most interest was the finding that normal glutamate-accumulating capacity of vitamin B₆-deficient cells could be maintained by high buffer tonicity (0.5 M sucrose). The author feels that the reduced accumulation may be related to the instability of a cell structure which is protected by high tonicity or repaired in the presence of acetate, NH₄⁺, and vitamin B₆.

The nature of trichloroacetic acid (TCA)-soluble derivatives of D-alanine in *S. faecalis* was inspected by Ikawa & Snell (56). Previous work had established that in the absence of vitamin B₆, D-alanine is essential for growth and that a majority of the D-alanine assimilated by the cells is present in the cell walls. In the present study, fractionation of the cold TCA extract from cells grown with D-alanine-1-C¹⁴ revealed that the bulk of the radioactivity was caused by free D-alanine (74 per cent) and D-alanyl-D-alanine (15 per cent). However, the picture in the case of the hot TCA extract was not quite as clear. Dialysis of the hot TCA extract yielded a dialyzable fraction that contained most of the radioactive D-alanine in an uncharacterized but non-nucleotide form, and a non-dialyzable fraction containing 39 per cent of the radioactivity of the extract and 12 per cent of the weight of the original dry cells. This component was found to contain the same amino acids and carbohydrates as the cell wall. It sedimented as a single substance in the ultracentrifuge with a molecular weight of 10,000 to 20,000. It is of interest in this regard to mention that Baddiley & Neuhaus (57) described the presence

of a specific D-alanine-activating enzyme in extracts of *L. arabinosus*. In addition to the probable importance of such an enzyme in the metabolism of D-alanine, the work represents the first example of an enzyme activating a D-amino acid.

A study of the effects of vitamin B₆ and its derivatives on diaminopimelic acid decarboxylase in *Bacillus sphaericus asporogenus* was made by Meadow & Work (58). They concluded that the data obtained are consistent with the view that a low affinity exists between this enzyme and its coenzyme in *B. sphaericus* and that the coenzyme required is probably pyridoxal phosphate.

A detailed study of the vitamin B₆ requirements of *Saccharomyces carlsbergensis* revealed a demand for this vitamin for rapid growth in the absence of added thiamine [Morris, Hughes & Mulder (59)]. The rate rather than the total amount of growth of the organism was found to be proportional to the concentration of the vitamin. Various conditions affecting the assay of vitamin B₆ with this organism were discussed as well as its use for the microassay of this vitamin. Sakuragi & Kummerow (60) investigated the behavior of ten antivitamin B₆ compounds in the nutrition of *S. carlsbergensis*.

The interrelationships of serine, glycine, and vitamin B₆ in the growth of mutants of *E. coli* were carefully inspected by Morris & Woods (61). The growth of two of these mutants, which exhibited suboptimal response to vitamin B₆, was more rapid and extensive when serine or glycine was present in the medium. These strains also grew in the absence of added vitamin B₆ when either glycine or serine plus glycolaldehyde was provided. In a subsequent paper, experimental evidence was given to support the conclusion that glycolaldehyde and serine or glycine are concerned in the biosynthesis of vitamin B₆ by these microorganisms, rather than acting as products of vitamin function [Morris (62)].

Vitamin B₁₂.—DeMoss & Happel (17) studied the nutritional requirements of *C. violaceum* with the finding that either D- or L-methionine or vitamin B₁₂ was required for growth. The fact that both enantiomorphs of the amino acid were active suggests the participation of a methionine racemase in this organism. Under certain conditions both violacein production and growth were found to be linear functions of the concentration of vitamin B₁₂ in the medium. On this basis, the organism may be useful for the microbiological assay of vitamin B₁₂, using growth or pigment production as a parameter of response.

Interrelationships among vitamin B₁₂ and methionine and its analogues both in growth of *E. coli* and in bacteriophage synthesis, were reported by Lockingen, Humphrey & Wyss (63).

A microbiological study of mono-substituted vitamin B₁₂ amides was carried out using *Lactobacillus leichmannii*, *E. coli* (B₁₂ mutant), *Euglena gracilis*, and *Ochromonas malhamensis* [Baker *et al.* (64)]. In contrast to other studies designating them as antivitamin B₁₂ compounds, the present workers found the methylamide, ethylamide, and anilide of the monocarboxylic acid of vitamin B₁₂ to be inactive as B₁₂ antagonists. Indeed, they satisfied the B₁₂ requirement of *E. gracilis* and *L. leichmannii* but not that of *E. coli* and *O.*

malhamensis. Combinations of suboptimal levels of methionine plus the methylamide or ethylamide derivatives gave greater growth of *E. coli* than this level of methionine alone. The reason for this was not elucidated.

A most important contribution was the finding that pseudovitamin B₁₂ appears to function as a coenzyme for the system converting glutamate to β -methylaspartate in cell-free extracts of *Clostridium tetanomorphum* [Barker, Weissbach & Smyth (65)].

Continued study of the role of vitamin B₁₂ in protein biosynthesis and, especially in amino acid activation, was reported by Wagle, Mehta & Johnson (66) using rat liver microsomes.

Pearlman & Barrett (67) studied the biosynthesis of cobalamins by washed cell suspensions of propionibacteria and streptomycetes. Significant amounts of cobalamins were synthesized quite rapidly by these organisms upon the addition of approximately 1 p.p.m. of cobalt salts.

Folic acid.—A review of folic acid coenzymes in metabolic reactions involving "active formate" and "active formaldehyde" has appeared [Huenekins, Osborn & Whiteley (68)].

A relationship between folic acid and riboflavin biosynthesis in *S. faecalis* was reported by Clapper & Meade (16), and a relationship between purines and folic acid vitamins in *Gaffkeya homari* was studied by Aaronson & Rodriguez (69).

The requirements for the conversion of pteroylglutamic acid to the citrovorum factor by preparations from *L. casei* were described by Heisler & Schweigert (70).

Campbell & Sniff (71) established the folic acid requirement of 20 strains of *Bacillus coagulans* as well as discussing the interrelationships between this vitamin and other members of the folic acid group and vitamin B₁₂. Nutritional factors affecting the biosynthesis of folinic acid by *E. coli* were described by Miller & Bond (72).

Lipoic acid.—Considerable progress has been made towards the elucidation of acetate-replacing factors. For most microorganisms, lipoic acid (thioctic acid) or mevalonic acid replace the stimulatory activity of acetate. However, lipoic and mevalonic acid are inactive for certain lactic acid bacteria which require acetate for growth. MaciasR (73) has studied *Lactobacillus delbrueckii*, inducing an acetate requirement by the addition of ferrous ions. Under such conditions the requirement for acetate could be replaced by any of several purine or pyrimidine nucleosides and nucleotides. Uridine and adenosine were the most effective, each exhibiting about 30 times more activity than acetate. Several hypotheses were offered to explain these results, one of which is the possibility that the activity or production of the nucleosides or nucleotides is antagonized by ferrous ion.

The effect of thioctic acid analogues on the growth of microorganisms and on certain bacterial enzyme systems concerned in acetate generation or transfer, was reported by Broquist & Stiffey (74) and by Albrecht & Broquist (75), respectively.

On the basis of studies of the pyruvic dehydrogenase system in myco-

bacteria, Goldman (76) suggested the presence of another form of lipoic acid; namely, lipoamide, which may be structurally closer to the true coenzyme form than lipoic acid itself.

Pantothenate.—A paper by Brown (77) provides an excellent comparative study of the metabolism of pantothenic acid. The results of this investigation establish that the major (and perhaps sole) pathway of coenzyme A biosynthesis in *Proteus morganii*, *E. coli*, rat liver, and rat kidney is as follows: pantothenic acid \rightarrow 4'-phosphopantothenic acid \rightarrow 4'-phosphopantothenylcysteine \rightarrow 4'-phosphopantetheine \rightarrow coenzyme A (CoA). The generally accepted pathway was considered not to be established unequivocally until the presence of an enzyme synthesizing pantothenylcysteine from pantothenic acid and cysteine can be demonstrated. This pathway consists of pantothenic acid \rightarrow pantothenylcysteine \rightarrow pantetheine \rightarrow 4'-phosphopantetheine \rightarrow dephospho-CoA \rightarrow CoA. Comparisons of the present work with that of Hoagland & Novelli (78) were presented.

The quantitative microbiological assay for bound forms of pantothenic acid in a number of microorganisms was reported by Brown (79) in a subsequent paper. Pantothenic acid, phosphopantothenic acid, pantetheine, phosphopantetheine, and CoA were determined. The results revealed that CoA was the major pantothenic acid-containing compound present, but small amounts of pantetheine and phosphopantothenic acid and larger amounts of phosphopantetheine were also found. Thus, CoA is not the only bound form of pantothenate present in cells. The presence of substantial quantities of pantetheine and phosphopantetheine suggests that they may be used as coenzymes in some metabolic reactions.

Para-aminobenzoic acid (PAB).—Reed, Schramm & Loveless (80) demonstrated that PAB in a concentration of 25 μ g./ml. or greater inhibited the growth of *S. cerevisiae*. Aromatic amino acids were effective in reversing this inhibition with the order of effectiveness being phenylalanine > tyrosine > tryptophan. In the presence of inhibitory concentrations of PAB, shikemic acid accumulated in the culture medium. The authors suggested that PAB may inhibit the utilization of shikemic acid or a subsequent common precursor of the aromatic amino acids. In contrast to the findings of Davis (81) with *E. coli*, *para*-hydroxybenzoic acid and shikemic acid were ineffective in reversing the PAB inhibition of *S. cerevisiae*.

Biotin.—The last few years have witnessed a renewal of activity in the biotin field with several reports of more than casual interest and importance. Perhaps the most important factors hindering rapid progress in the elucidation of the enzymic functions of biotin are the lack of availability of the active form of the vitamin and its firm attachment to enzyme proteins. With respect to the first point, little progress has been made, and it becomes obligatory to employ certain natural products as convenient, albeit complex, forms of the biotin coenzyme or to employ intact cells capable of converting biotin to an active form. With regard to the second point, it is becoming increasingly clear that a severe biotin deficiency is required if one wishes to obtain cells exhibiting reduced enzyme activity.

An x-ray crystallographic analysis of the molecular structure of biotin has suggested that the vitamin may be capable of forming an intramolecular hydrogen bond in solution [Traub (82)]. Thus, the proposed mechanism of hydrogen transport as a mode of action of biotin [Lichstein (83)] is strengthened by these physical studies, although the actual mechanism differs somewhat.

A site of action of biotin in glycolysis appears to be in the hexokinase system. Williams, Andrews & Christman (84) studied glucose and 2-deoxy-D-glucose utilization by intact cells and cell-free extracts of biotin-deficient *S. cerevisiae*. 2-Deoxy-D-glucose was employed because it can be phosphorylated by yeast hexokinase and cannot be further metabolized. Sonic extracts of biotin-sufficient yeast were found to phosphorylate 2-deoxy-D-glucose at an appreciably faster rate than those obtained from biotin-deficient cells. Unfortunately, attempts to reactivate the deficient preparations with biotin were unsuccessful. Thus, although the site of biotin action in glycolysis was more precisely localized, the mode of action was uncertain. A more direct study of the hexokinase system in the same organism was made by Strauss & Moat (85). Hexokinase was measured by coupling it with glucose-6-phosphate dehydrogenase and measurement of TPN reduction at 340 m μ . The pertinent findings suggesting a direct effect of biotin on the hexokinase reaction were stimulation of glucose and fructose fermentation by biotin, the lack of biotin stimulation of fermentation of glucose-6-phosphate, fructose-6-phosphate and hexosediphosphate, biotin stimulation of hexokinase activity in dried cells and cell-free extracts, and the lack of stimulation of the phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase. Of considerable interest was the finding that cell-free extracts required no preincubation with biotin for stimulation in contrast to the requirement of a 30- to 40-min. preincubation with dried cells in order to obtain stimulation. Since hexokinase can be obtained in crystalline form, it will be interesting to know whether or not biotin is present in the enzyme as revealed by microbiological assay. Recent instrumental developments have made possible the extension of the microbiological assay method for biotin in a scale permitting, without loss in accuracy or precision, use of 1/500 to 1/1000 as much sample as in the conventional assay procedure [Glick *et al.* (86)]. Such a method might well be employed to determine the biotin content of crystalline hexokinase isolated from biotin-deficient and biotin-sufficient yeast cells.

During studies on the nutritional requirements for biological nitrogen fixation by *Clostridium pasteurianum*, Carnahan & Castle (87) noted substantially higher requirements for biotin and for iron when the organism was fixing nitrogen than when assimilating ammonia. Also, biotin-deficient cultures could be stimulated with ammonia, but ammonia was not stimulatory to cultures fixing nitrogen in the presence of adequate biotin. Although the role of biotin in nitrogen fixation remains to be identified, it is noteworthy that this vitamin is associated in some manner with another important biological process.

Sund, Ravel & Shive (88) demonstrated that biotin-deficient cells of

Streptococcus lactis exhibit a greatly reduced ability to convert ornithine and carbamyl phosphate to citrulline. The requirements for restoration of activity (incubation with glucose, amino acids, glutamine, phosphate, and biotin) and the fact that this restoration was inhibited by certain purine and pyrimidine analogues suggest that *de novo* synthesis of the ornithine-citrulline enzyme is carried out in the presence of biotin. In a subsequent paper, the enzyme was isolated and purified from cell-free extracts of *S. lactis* [Ravel *et al.* (89)]. Using *S. cerevisiae* as assay organism, biotin was estimated in the purified preparations and in the original cell extract after acid hydrolysis. No comparison was made between biotin-sufficient and biotin-deficient preparations. On the basis of their results it was concluded that biotin is not a component of the enzyme, unless the vitamin is present in the enzyme in a form which does not yield activity upon acid hydrolysis. In a more detailed account, these workers arrived at the same conclusion, namely, that biotin exerts its action during the synthesis of the ornithine-citrulline enzyme and is probably not a component of the enzyme (90).

The specificity of biotin in the metabolism of propionibacteria was inspected by Lichstein (91). The organism chosen was *Propionibacterium pentosaceum* and the systems studied were succinate decarboxylase and glucose fermentation. Preliminary results revealed that biotin and dethiobiotin stimulated the succinate decarboxylase system of biotin-deficient intact cells, while oxybiotin possessed only limited ability to replace biotin for this system. Further study demonstrated that cells harvested from media in which biotin was replaced by dethiobiotin exhibited the same succinate decarboxylase activity, suggesting that dethiobiotin was capable of replacing biotin for this enzyme system. However, cells grown in media containing oxybiotin in concentrations supporting growth equivalent to that obtained with biotin possessed very low succinate decarboxylase activity. Furthermore, the reduced succinate decarboxylase activity of oxybiotin-grown cells did not appear to be related to apoenzyme content but rather to a deficiency in cofactor since activity was restored fully by the addition of biotin. In sharp contrast to the results with succinate decarboxylase, it was found that bacterial cells harvested from media containing biotin, oxybiotin, or dethiobiotin exhibited the same ability to ferment glucose. Although these studies deal with ill-defined functions of biotin, it is clear from the results that both systems require biotin for optimal activity in the intact cell. It is also quite clear that biotin and dethiobiotin are mutually replaceable for both glucose fermentation and succinate decarboxylase, whereas oxybiotin can replace biotin in the former system but substitutes for biotin only to a limited extent for the latter. It was suggested that oxybiotin might be converted into an oxybiotin coenzyme which has relatively poor ability to serve as a cofactor for the succinate decarboxylase system but is fully capable of replacing the biotin coenzyme in the fermentation of glucose. With regard to dethiobiotin it is possible that it may function directly in both systems or after conversion to biotin. Thus, the possibility of the existence of more than one active form of biotin was presented as a working hypothesis which obviously requires

extensive experimental work before establishing. Furthermore, it appears entirely possible that studies with homologues and analogues of biotin may contribute to a better understanding of the mode of action of this vitamin in enzyme systems.

The permeability of cells of *L. arabinosus* to biotin was studied by Lichstein & Ferguson (92). The experimental design consisted of suspending biotin-deficient cells in a solution of biotin buffered at pH7. After incubation at 37°C. the cells were recovered, washed thoroughly and hydrolyzed with H_2SO_4 . The level of biotin in the deficient cells varied from $1.4\text{--}1.9 \times 10^{-4} \mu\text{g.}$, and after incubation with biotin the range found was $5.8\text{--}18.4 \times 10^{-4} \mu\text{g.}$ The addition of glucose in the presence of added biotin resulted in increases of two- to fourfold and significantly the effect of glucose was inhibited completely by the presence of iodoacetate. Furthermore, the presence of homobiotin in appropriate concentrations resulted in complete inhibition of biotin uptake by the bacterial cells. Thus, the results are in keeping with the concepts of active transport and stereospecific permeation systems. However, further work is required to distinguish between membrane permeability or adsorption to active sites within the cell. Regardless of this, it appears reasonable to assume that studies on the permeability of bacterial cells to biotin and other B vitamins will be of value in providing a better understanding of the physiological function of these compounds.

Two interesting papers concerned with fatty acid stimulation of bacterial growth and its relationship to biotin have appeared. The first by O'Leary (93) is concerned principally with the utilization of octadecenoic acids by *L. arabinosus* grown in media containing *cis*-vaccenic-1- C^{14} or oleic-1- C^{14} acid in place of biotin. The constituent fatty acids of these harvested cells were separated chromatographically. Most of the fatty acid removed from the medium could be accounted for by the amount of octadecenoic and lactobacillic acids which constitute the major cellular content of these acids. The author claims that these observations support the concept that biotin is involved in the synthesis of unsaturated fatty acids essential to the cell. However, no direct evidence of such a pathway was presented. Similar studies with similar interpretations were presented by Hofmann *et al.* (94). This reviewer has no quarrel with either the excellence of this work or the interpretation given to the results, and certainly there is increasing evidence for the participation of biotin in fatty acid synthesis [see, for example, Woessner, Bachhawat & Coon (95) for exhaustive literature references]. However, they provide no answer to the fact that fatty acids exert a sparing effect on riboflavin and pantothenic acid as well as biotin when employing certain strains of lactobacilli for microbiological assay. Indeed, it was shown recently that oleic acid enhances the activity of thiamine for *Lactobacillus fermenti* [MaciasR (96)]. Furthermore, unless the growth medium is completely free of biotin it is possible that some of the fatty acid effect may be related to surface activity properties which may facilitate the entrance of residual biotin into the cells [Traub & Lichstein (97)].

Katsuki (98) observed a marked diminution in the amount of ATP and

pyridine nucleotides in the mycelia of biotin-deficient *Piricularia oryzae*, suggesting that biotin may have some function in the synthesis of these compounds. This aspect was investigated further in *Bacillus macerans* (99). Perhaps the most interesting findings were that the decreased activity of α -keto acid oxidation could be restored by DPN or ATP and that the efficiency of oxidative phosphorylation decreases under biotin deficiency.

Dhyse & Hertz (100) have revived the vitamer concept of Burk & Winzler (101). The significance of their findings is not yet clear except to re-emphasize the existence of many natural forms of biotin.

A brief paper by Ferguson & Lichstein (102) presents comparative data on the biotin content of *Bacillus terminalis* as determined by microbiological assay employing four different assay organisms. The range of values obtained emphasizes the complexities inherent in the microbiological assay method for biotin and the difficulty of obtaining the true biotin content of a sample.

Maretzki & Guerrant (103) studied the influence of biotin on protein formation in *L. casei* and *L. arabinosus* with the finding that there was no positive effect of concentrations of biotin in excess of that required for optimum growth. However, total lipid formation was affected by the biotin concentration. The biotin requirements of a methanol-utilizing strain of *Pseudomonas* was studied in detail by Kaneda & Roxburgh (104).

The effect of isoniazid on the synthesis of certain amino acids and vitamins by *Mycobacterium tuberculosis* was investigated by Willett (105). Significantly larger quantities of biotin were present in the resistant organism. Cultivation in the presence of subinhibitory concentrations of isoniazid resulted in larger amounts of biotin in both susceptible and resistant strains. It is not clear whether the larger quantities of biotin were the result of increased synthesis or decreased utilization or conversion to a bound form.

The reader is referred to the papers of Lynen (146), Swick & Wood (147), and Stadtman *et al.* (148) for current developments.

Inositol.—A stimulating paper concerned with unbalanced growth of yeast caused by inositol deficiency was published by Ridgway & Douglas (106). The pertinent finding was that cells of *S. carlsbergensis* or *Kloeckera apiculata*, when grown in inositol-deficient media, rapidly lost viability after an initial period of multiplication albeit the total number of cells continued to increase. Further experiments provided proof that growth was necessary for death to occur as a result of inositol deficiency. It was also demonstrated that the inositol-deficient cells were low in cytochrome, coenzyme A, and DPN. As a working hypothesis the authors suggest that the basic lesion involved in the described unbalanced growth is one affecting the structural integrity of cytoplasmic particles, possibly the mitochondria. The authors had demonstrated in an earlier paper that in yeasts inositol is concentrated in the cytoplasmic particles of the cells (107).

Vitamin K.—In contrast to the documented studies revealing the ability of many microorganisms to synthesize vitamin K, only a few species have been reported which require this vitamin for growth. The growth-promoting activity of compounds of the vitamin K group for *Fusiformis nigrescens* was

studied by Lev (108) and compared with those from animal assay techniques. With two main exceptions, the compounds tested possessed the same relative activity for the microorganism and the animal. 1:4-Naphthoquinone was active for *F. nigrescens* and inactive for animals, while phthiocol, which is active for animals, acted as an antivitamin K for the microbe. The results suggest the possibility that a practical microbiological assay for the vitamin K group might be developed. Glick, Zilliken & György (109) have demonstrated the growth-stimulating effect of 2-methyl-1,4-naphthoquinone (menadione) on *L. bifidus* var. *pennsylvanicus*.

On the basis of studies employing particulate and soluble fractions from *Mycobacterium phlei*, Weber, Brodie & Merselis (110) suggest a role for vitamin K in electron transport. Their results suggest that the oxidation of reduced DPN by the bacterial particles proceeds via two pathways. One proceeds through the cyanide-sensitive terminal respiratory pathway and involves vitamin K, or a related compound. The other involves a diaphorase-type, flavoprotein-catalyzed, cyanide-insensitive reaction.

The natural occurrence of coenzyme Q and related compounds was surveyed by Lester & Crane (111) in a number of biological species. The coenzyme was found to be widespread but not ubiquitous. In general, its amount and intracellular distribution were correlated with aerobic respiratory capacity, but several outstanding exceptions were noted as, for example, its absence in *M. tuberculosis*.

Miscellaneous.—An excellent and provocative review on microbiological assays has appeared [Hutner, Cury & Baker (112)]. An interesting correlation between the methionine content of casein hydrolyzates and certain difficulties in the microbiological assay of vitamins has been made by Brodovsky, Utley & Pearson (113). Using two strains of *S. faecalis*, they found a direct relationship between the methionine concentration of the acid-hydrolyzed caseins and their ability to support growth. Considerable variation was found in methionine content among several batches of commercial products. The authors now supplement their folic acid assay media with methionine and they recommend that the possible inadequacy of the usual amounts of casein hydrolyzate to meet the amino acid requirements of certain organisms should be considered as a source of difficulty with microbiological assays.

The requirement of a mixture of five coenzymes and glucose-1-phosphate to replace the ascitic fluid normally required for proliferation of *Borrelia vincentii* was reported by Hampp & Nevin (114). The coenzymes needed were cocarboxylase, codecarboxylase, coenzyme A, DPN, and ATP. These unusual requirements must reflect poor synthetic ability by this organism as well as a permeability mechanism for compounds not ordinarily capable of penetrating microbial cells. In a subsequent paper (115), they reported that the amount of ascitic fluid needed for maximal growth could be reduced from 10 per cent to 0.3 per cent by the addition of coenzyme A, ATP, NaHCO₃, and L-asparagine.

The successful selection and isolation of auxotrophic yeast mutants with the aid of antibiotics (amphotericin B, endomycin, and nystatin) was reported by Moat, Peters & Srb (116). The limitation of the method was the survival of respiratory-deficient yeast mutants in the presence of the antibiotics employed. These studies represent a logical extension of the penicillin selection method for bacterial auxotrophs.

MISCELLANEOUS

Stokes & Bayne (117) have made a thorough study of the growth factor requirements of six strains of the genus *Salmonella*. Whereas most strains of this genus have simple nutritional requirements, a number have been isolated which do not synthesize certain vitamins and amino acids. These authors found the growth factor requirements to include the vitamins thiamine and nicotinic acid, the purines adenine and guanine, and the amino acids cystine, methionine, leucine, threonine, histidine, arginine, and aspartic acid. On the basis of these results, consideration should be given to the nutritional inadequacy of most media employed for the primary isolation of *Salmonellae*.

A comprehensive study of the lipid requirements for the growth of pleuropneumonia-like organisms has been reported by Smith & Lynn (118). Certain strains have an obligatory requirement for a sterol and a phospholipid or surface active agent in addition to a protein substance. Cholesterol was found to satisfactorily supply the sterol requirement with optimal growth in the presence of approximately 3×10^{-6} M. Certain fatty acid esters of cholesterol and sterols with only minor modifications from cholesterol in the side chain and ring structure supported growth. It was ascertained that the 3-hydroxy group, the ring structure, and the intact side chain are required for growth. The lecithin requirement could be met in part with sodium cholate but not with cephalin or the component parts of a lecithin. The presence of acetate was stimulatory for growth.

Demain, Hendlin & Newkirk (119) have contributed to our understanding of the nutritive value of vitamin-free digests of casein. During studies on the nutritional requirements of a species of *Sarcina* they noted a marked shortening of the lag phase when the synthetic medium was supplemented with vitamin-free hydrolyzates of casein, but not by the unhydrolyzed material. Further investigation revealed a requirement for long-chain fatty acids rather than a peptide as might have been suspected. The most potent compounds tested were long-chain unsaturated fatty acids, while of many saturated acids examined only lactobacillic acid was active. The results demonstrate the danger of using casein digest media for the study of the fatty acid nutrition of microorganisms. MacLeod & Morgan (120) reported the isolation of α -ketoglutaric and pyruvic acids as their 2,4-dinitrophenylhydrazones from five different commercial samples of vitamin-free acid hydrolyzates of casein. Also, one sample yielded traces of α -ketobutyric acid. These findings are important in any nutritional study in which acid-hydrolyzed casein provides the source of amino acids. Both studies reveal the pitfalls in assuming

that hydrolyzed casein serves only as a source of amino acids and peptides.

The growth-stimulating effect of autoclaved glucose media on propionibacteria has been studied by Field & Lichstein (121) with the conclusion that there is a relationship between this phenomenon and the carbon dioxide requirement for growth initiation of these organisms from small inocula. It will be interesting to determine if carbon dioxide can replace the growth-stimulatory effect of heated glucose noted in a variety of other microorganisms [Guirard (122)]. In this connection, Demain & Hendlin (123) studied the nutritional requirements of *Microbacterium lacticum* with the finding that the growth of this organism was stimulated by low concentrations of ferriochrome, coprogen, or terregens factor which are thought to function as intracellular "iron transport factors" [Neilands (145)]. Of interest was the activity of glucosylglycine which had been shown earlier to replace the autoclaved glucose effect in *Lactobacillus gayoni* [Rogers, King & Cheldelin (124)], and *Propionibacterium freudenreichii* [Field & Lichstein (125)].

Several papers have appeared on the nutrition of acid-fast microorganisms. For example, Vogel (126) investigated the nutritional requirements of 11 strains of acid-fast bacteria isolated from cold-blooded animals. Perhaps the most striking finding was the marked stimulation of growth of nearly all strains by the presence of the diethyl and γ ethyl ester of L-glutamic acid. The author postulates that the failure of fastidious strains of acid-fast organisms to grow may be caused by the inability of certain substrates, particularly polar compounds, to penetrate the waxy and lipoidal surface of the cell. Inasmuch as the glutamate esters are less polar than the free amino acid they may pass through the cell membrane more readily. It will be important to extend these studies to include the mammalian strains of tubercle bacilli. Aoyagi & Mizuno (127) studied the submerged diffuse growth of several strains of mycobacteria in a chemically defined medium containing a high concentration of Tween 80 but without the usual addition of albumin to counteract the toxic effect of free oleic acid possibly present in the Tween 80. Employing a fairly large inoculum (0.3 to 0.5 mg. dry wt. organism/10 ml.) it was found that growth increased with increasing concentration of Tween 80 up to about 1 per cent. Using generation time as a criterion, they reported that asparagine and glucose were the preferred nitrogen and carbon sources, respectively. The reported stimulatory effect of vanadium ions on the growth of species of mycobacteria could not be confirmed by Costello & Hedgecock (128). Actually, these workers found that the dispersed growth of *M. tuberculosis* from small inocula was inhibited by microgram quantities of vanadium. Finally, Spitznagel & Sharp (129) observed that both magnesium and sulfate are required for characteristic growth and reproduction of *Mycobacterium bovis*. Changes in cellular and subcellular morphology caused by deficiencies in these ions were described.

In a continued study of the nutrition of *Fusobacterium nucleatum*, Omata (130) has shown the absolute requirement for pantothenic acid, tryptophan, and purines. Both pantetheine and coenzyme A were highly effective in pro-

moting growth although pantothenic acid itself was more efficiently used. While adenine was the most active of the purines tested, it is noteworthy that 4-amino-5-imidazole carboxamide possessed definite growth-stimulating activity.

With regard to mineral nutrition, Lester (131) has made a careful study of the potassium requirements of a number of bacterial species. The growth of all species examined was enhanced markedly by K^+ or Rb^+ , but not by Cs^+ when tested in a relatively simple synthetic medium. In most cases, Rb^+ served as a satisfactory substitute for K^+ . The K^+ requirement was just as pronounced in a complex medium containing many growth factors. It is pertinent that Cs^+ replaced partially the K^+ requirement for certain species when tested in the complex medium. The iron requirements of two strains of *Leptospira icterohaemorrhagiae* were examined by Faine (132). Using inocula less than 10^7 organisms/ml. in Korthof medium, growth did not initiate unless $FeCl_3$, haematin, or preparations containing iron porphyrins were added. Larger inocula grew without these additions possibly because of carry-over of iron or iron-containing compounds. These results fit in well with recommendations that media employed for the cultivation of these organisms contain haemolyzed erythrocytes or a solution of laked erythrocytes. The sensitivity of virulent and avirulent strains of *L. icterohaemorrhagiae* to NaCl in culture media was also reported [Faine (133)].

The growth of *Treponema pallidum* could be increased two to threefold by the addition of monoglyceride tartaric acid ester compounds to a thioglycolate broth medium supplemented with 10 per cent sheep serum [Power & Pelczar (134)]. These esters could be replaced by a mixture of palmitic, stearic, and oleic acids.

Goldschmidt & Taylor (135) studied the nutritional requirements for growth and arthrospore formation by the dimorphic fungus, *Coccidioides immitis*. A synthetic medium composed of glucose, ammonium lactate, and inorganic salts supported excellent growth, although the yield of cellular protein per ml. could be doubled by employing complex media. Factors concerned with fragmentation of the hyphae into arthrospores were considered. Some of the nutritional requirements for improved spherulation of *C. immitis* in a chemically defined medium were investigated by Converse & Besemer (136). Nutritional factors influencing the conversion of the mycelial phase to the yeast phase in *Histoplasma capsulatum* were reported by Pine & Peacock (137).

The utilization of amino acids as carbon sources by *Streptomyces fradiae* was studied by Romano & Nickerson (138). By use of quantitative microbiological assay procedures, Gerhardt & Ball (139) demonstrated the utilization of several amino acids by *Leptospira canicola* growing on Schüffner's medium.

An improved medium for the obligate chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans* that supports the growth of 2 to 4×10^8 cells/ml. was described by Silverman & Lundgren (140). A chemically defined medium

for the growth of *Diplococcus pneumoniae* was designed by Rappaport & Guild (141). A study of the nutritional requirements of the anaerobic cellulolytic coccus, *Ruminococcus flavefaciens*, was made by Ayers (142).

What appears to be the first demonstration that a member of the genus *Streptococcus* can utilize ammonium salts as the sole source of nitrogen was made by Wolin, Manning & Nelson (143), studying *S. bovis*. Okazaki & Okazaki (144) studied DNA synthesis and cell growth by a deoxyriboside-requiring strain of *Lactobacillus acidophilus*.

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INTRAMURAL SPREAD OF BACTERIA AND VIRUSES IN HUMAN POPULATIONS¹

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INTRODUCTION

When a child contracts measles from a schoolfellow, or an adult catches influenza from his neighbour in an office, how do the microbes pass from one host to the other? How do staphylococci reach surgical wounds in hospital wards, or newborn infants in the nursery? It is questions of this sort that are the subject of this review—not the sources of infection in intramural epidemics, but the mechanics of the spread of the pathogens from one person to another.

The routes by which microbes are spread are traditionally classed as: (a) by contact, direct and indirect; (b) by food and drink; (c) by insect vectors; and (d) by the air. Although the term "contact" has often been used to include close-range droplet infection through the air, it seems much better to confine the word to its commonsense meaning. This review will not deal with food- or insect-borne disease, but will be concerned with spread by contact and by the air, the latter being given special prominence because it has been so much more extensively studied.

The studies of air-borne infection, though extensive, are unsatisfactory in the sense that although the advances in techniques have been considerable we have hardly discovered how to apply these advances to the problem of human disease in normal communities. There is, therefore, a much less direct connection than would be desirable between the first, technical and experimental, part of this review and the second, epidemiological part.

The last 100 years have seen striking changes in the belief accorded to the existence of air-borne infection. Early faith in its importance was shattered by Flüge's demonstration that infected droplets from the mouth did not appear to travel more than two meters, and by Chapin's finding that diseases seemed not to spread when contact and direct droplet infection were limited. The idea of air-borne infection was, however, revived by Wells' technological advances in the 1930's and was the subject of great interest during the next two decades. It has perhaps subsequently suffered some decline from a realization of the size of the gap between laboratory discovery of a potential method of spread and field demonstration of its real importance.

The actual way in which the microbes that cause disease pass from one individual to another can only be discovered by a combination of clinical epidemiology and applied microbiology. The very diffuseness of air as an

¹ The survey of the literature pertaining to this review was concluded in November, 1959.

element makes the epidemiological study of aerial spread far more difficult than that of food-borne infection; and the microbiological techniques for the study of air-borne organisms require a far greater understanding of physics than is needed for more tangible media. Flügge's denial of air-borne spread derived from his lack of realization that his techniques were inadequate to demonstrate it, and Wells' later advocacy of the importance of air-borne spread suffered from his failure to produce epidemiological evidence to support it.

DESCRIPTIVE BACTERIOLOGY

AIR SAMPLING TECHNIQUES

Collection techniques.—Up to the present, practically no satisfactory methods have been devised for collecting living virus particles from the air so that this discussion is limited to bacterial air sampling. The simplest method of collecting air-borne bacteria is to allow them to settle onto exposed culture plates, and for many purposes the major limitations of this method—that it samples an unknown volume of air and that it must always give an undue emphasis to the bacteria carried on the larger particles—do not matter. These two limitations have, however, stimulated a great deal of work on various samplers designed to collect all the airborne particles, including the small ones, from a given volume of air. These volume samplers usually operate on the basis of filtration or impingement of the particles onto a solid surface or a liquid film.

Two types of impinger are now in general use: the slit sampler described first by Bourdillon, Lidwell & Thomas (17), and the fluid impinger devised at the Microbiological Research Establishment, Porton [e.g., Henderson (59); May & Harper (83); Rosebury (101)]. In the slit sampler the air is drawn through a narrow slit in the top of an airtight box and the emerging jet of air impinges on a culture plate rotating 2 mm. below the slit. The original instrument sampled air at 1 cu. ft. (28 litres) per min., as does a simplified slit sampler for field work described by Decker & Wilson (29). An adaptation made to sample at about 20 cu. ft. (560 l.) per min. [Bourdillon *et al.* (16)] has proved invaluable when pathogenic bacteria are sought. One of the great advantages of the slit sampler is its ability to yield a time-discriminated sample by arranging that a particular area of the agar passes under the slit only once. For long-period sampling, several modifications have been described, including the spiral-track sampler giving a 20-in. long track on a 5½-in. diameter Petri dish [Lidwell (70)], and a moving plate sampler [Decker *et al.* (28)] in which a long rectangular plate travels under a slit. The last incorporates a device for transferring the exposed plates to an incubator, thus making use of the other great advantage of the slit sampler, the fact that the sample is collected directly onto the culture plate.

When the air contains very large numbers of bacteria, as is often the case in experimental work, collection directly onto the agar surface is a disadvantage; in this case the microbes need to be collected on some material from

which they can be subsequently suspended in fluid and diluted. Although various "bubbler" samplers have been used in the past the most satisfactory instrument collecting directly into fluid appears to be the critical-orifice impinger. Air is sucked at approximately 130 cm. sec. through a 1.1 mm. jet held 30 mm. above the surface of the collection fluid, under half an atmosphere vacuum; the jet acts as a critical orifice and automatically governs the air flow to about 11 litres per min. Cown, Kethley & Fincher (24) studied a similar impinger with a 0.33 mm. orifice working at 1.0 litre per min.

Another sampling method that has received attention is filtration. Membrane filters have been used by several workers [e.g., Albrecht (1)] but the volume of air that can be handled is small and the risk of desiccation of the bacteria collected on the filter seems likely to be large; Albrecht found membrane filters unsuitable for sampling naturally contaminated air. Soluble filters have been made of many substances including calcium alginate [Richards (99)], sodium glutamate [Vanini (114)], and fibrin foam [Noller & Spendlove (87)]. The last were unsatisfactory for sampling *Chromobacterium prodigiosum* clouds, though less so for spore clouds.

Lastly, electrostatic precipitator samplers have also been used to a small extent [e.g., Houwink & Rolvink (61); Luckiesh, Holladay & Taylor (80)]. They have the great advantage of low resistance to air flow, but difficulties arise from the fact that not all bacteria-carrying particles are precipitated in either the positive or the negative field.

Experimental work in air-borne infections has demonstrated the importance of the size of infective particles in governing their behaviour. In the cascade impactor described by May (82) for sizing the particles in dust clouds, air is drawn through a series of jets, successively decreasing in width, and is accelerated at each stage so that successively smaller particles are impinged on a series of four slides. The same principle has been adapted for determining the size of bacteria-carrying particles in a six-stage sieve sampler [Andersen (4)] in which the diameter of the sieve holes decreases at each stage; particles over 8.2 μ in diameter are collected on the first, and particles below one micron on the sixth.

A four-stage slit sampler working at 20 cu. ft. per min., which overcomes the inherent difficulties of a sieve sampler, was described by Lidwell (71). Four slits varying between 1.1 and 0.05 mm. in width impinge particles (over 18, 10 to 18, 4 to 10, and below 4 μ in diameter) onto four 15 cm. diameter Petri dishes.

It is not, however, always sufficient to know the size of the air-borne particles; we are also interested in the number of viable units they contain. Some estimate of this can be made by comparing the number of colonies obtained when the particles are collected directly on a solid medium with the number obtained by collection into liquid, in which particles may be expected to be broken up. With naturally occurring bacterial clouds, however, the break-up of the particles is not easy and is in any case difficult to

determine. Lidwell, Noble & Dolphin (74) overcame these difficulties by exposing culture plates seeded in the size-grading sampler to varying doses of high-intensity electron bombardment in a linear accelerator. By application of multiple-hit theory they were able to estimate the average number of viable units per colony-forming particle.

Survival of collected bacteria.—Much less attention has been given to the fate of the bacteria after collection than to methods of separating them from the air. May & Harper (83) obtained evidence that excessive turbulence in an impinger sampler could kill vegetative organisms; Goldberg & Schechmeister (47) found the same for excessive speed of impingement in a slit sampler. Cown, Kethley & Fincher (24) showed that survival of vegetative organisms was best when they were collected into a nutrient medium, though in practical sampling this may offer the risk of multiplication of the bacteria between collection and plating. The complex inter-relations between temperature and humidity in sampling clouds from chambers held at various temperatures were studied by Kethley, Fincher & Cown (66). The great disadvantage of the filter samplers seems to be the death of the bacteria on the filters, presumably through drying from the stream of air. The same phenomenon may be seen with the slit sampler when the plate travels so slowly under the slit, that there is excessive local drying of the agar [see Kuehne & Decker (68), amply confirmed by unpublished work in this laboratory].

Selection of particular microbes.—There is no need to discuss here the various bacteriological media used to separate the microbes of interest from the "general" bacterial flora of the air, but one fact needs to be recalled. It seems likely that selective media often have a slight inhibitory effect even on the bacteria that they are designed to select, and this effect may be much more marked with bacteria collected from the air than it is on laboratory cultures used to test the media [e.g., Williams & Hirsch (123)].

"Indicator" organisms.—A recurrent problem in studying the transmission of disease by bacteriological methods is the difficulty of demonstrating the actual pathogenic microbe in the environment. This results not only from the frequent technical difficulties in cultivation—as with the viruses, the tubercle bacillus, etc.—but also from the small numbers that are usually present in a room even when they are being liberated freely by a patient or carrier. The pathogenic bacteria that have been detected in natural environments include the pyogenic cocci, *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, and *Escherichia coli*. But the diseases whose spread has been regarded as air-borne include many virus infections of the respiratory tract such as the common cold and influenza, and generalized infections like measles and chickenpox. The idea was therefore developed, first by Gordon (48), that some normal commensal bacteria of the respiratory tract of the occupants of a room might be enumerated to indicate the amount of respiratory tract pollution of the air, and therefore the potential risk of pollution with pathogens if a dangerous carrier should be present, in the same way as

E. coli is used to indicate pollution of water. The commensal mouth streptococci (*Streptococcus* "mitis" or "viridans" and the levan-producing "*salivarius*") have been used.

It cannot, however, be taken as axiomatic that the number of mouth bacteria in the air is necessarily an index of the hygienic adequacy of the ventilation in a room. To complete the analogy with the use of *E. coli* as a hygienic index for water supplies, we have to show that the only significant source of the mouth bacteria in the air is the respiratory tract of human beings, that the commensal bacteria are dispersed in the same way and survive for much the same time as the pathogens, and that human disease results from spread of these pathogens by the same routes as the indicator organisms. Or we have to demonstrate that there is, in fact, a correlation between some bacterial count and the spread of disease.

Sources of bacterial flora of air.—In several recent studies an attempt has been made to define the sources of the air-borne bacteria, and the factors affecting their numbers. Thus, Williams, Lidwell & Hirsch (125) demonstrated a correlation of the *Streptococcus salivarius* count in schoolrooms with the amount of talking among the occupants, and concluded that the mouth was the principal source of these bacteria. The general bacterial count (i.e., total aerobic flora cultivated on serum agar incubated at 37°C.) was less highly correlated with talking, but was correlated with the activity of the occupants of the room. The general count was reduced when the room ventilation increased, as in warm weather when windows were opened; but increased ventilation had no effect on the streptococcus count. This difference was attributed to the greater mean size of the streptococcus-carrying particles (as determined by comparison of a volume-count and the settling count); the larger particles have the more rapid settling rate and can therefore be less affected by the dilution effect of increased ventilation.

As a rule, in occupied places the general bacterial count seems to be related to the amount of disturbance of fabrics: Williams & Hirsch (124) found that it was higher at the time when people entered an underground railway train than at a time when the car was full and most people were motionless. In schools, the count was related to "activity" by the room occupants [Williams *et al.* (125)]. In hospital wards, bedclothes and bedcurtains seem to be the important sources and bedmaking the most potent disperser [Bourdillon *et al.* (16)]. In hospital operating rooms, the disturbance associated with the patient's entry and with disturbance of his bedding and any wound dressings are important [Bourdillon & Colebrook (14)] but activity by the surgical team also leads to high counts [Blowers (10)].

Staphylococcus aureus is one of the pathogens that can be found in the air, and recent interest in staphylococcal hospital infection has prompted studies of its prevalence. In a surgical ward [Shooter *et al.* (107, 108)], we found that high staphylococcus counts were to be expected when many patients were infected and there was extensive contamination of fabrics. But, apart from this, some individuals seemed to be particularly apt to

broadcast their staphylococci. In the ward some of these dispersers were patients with lesions that led to abundant contamination of bedclothes, but other equally active dispersers apparently shed their staphylococci from healthy sites onto their bedding. These observations were very similar to earlier findings with hemolytic streptococcal infection, in this case the active disperser being the heavy nasal carrier [Hamburger, Green & Hamburger (50)].

SURVIVAL OF MICROBES IN THE ENVIRONMENT

The idea that microbes may be conveyed from one host to another by droplet nuclei or by way of contaminated dust, naturally stimulated studies on the length of time that microbes can survive outside their host. [Extensive lists of references to published work on survival of microbes have been published by Engley (41, 42).]

There have been two approaches: some have studied the survival of naturally dispersed material, and others that of artificially dispersed laboratory cultures. Laboratory cultures afford opportunity for carefully controlled quantitative experiments but at the cost of the extrapolation needed to apply the results to natural situations. The study of natural dust involves less extrapolation but suffers from the lack of control on exact numbers and state of the organisms initially present.

Attempts have also been made to study the survival of microbes in airborne clouds either in continuous flow systems [Kethley, Fincher & Cown (66)] or with methods involving storage of the aerosol in bags or spheres [Ferry *et al.* (43)]. All of these methods suffer more or less from the difficulty encountered in distinguishing between a decrease in bacterial count resulting from deposition of the organisms on the floor of the chamber and a decrease caused by their death. The settling rate can, however, be measured by mixing radioactive killed organisms with the cloud of living organisms under study; preferably, the same bacteria or spores are used [Harper, Hood & Morton (54)]. Settling can be measured accurately with the radioactive bacteria, and the same rate can be assumed to apply to the living bacteria. The difficulties caused by settling have also been overcome by a recent elegantly simple method in which the cloud is not allowed to settle because it is held in a drum rotating at such a speed that the particles fail to impinge on the walls [Goldberg *et al.* (46)].

Humidity is the factor most studied in relation to survival in aerosols. Dunklin & Puck (40) showed that sprayed cultures of pneumococci were most rapidly killed at humidities around R.H. 50 per cent. This effect is apparently attributable to the salt present in the culture medium which becomes concentrated around the cell during drying. It is thought that at some humidities this process of concentration is lethal. A very complex set of relations between temperature and humidity was observed with *Chromobacterium prodigiosum* in a continuous flow apparatus by Kethley *et al.* (65, 66), who noted that the results obtained varied both with the medium

from which the bacteria were sprayed and also with the material into which they were collected. The bactericidal effect of light also apparently varies with humidity; [Beebe (7); Beebe & Pirsch (8)]. Several workers [Ferry *et al.* (43, 44)] have found that in the death of air-borne bacteria of various species two stages can be recognized: the first rapid and the second much slower.

Practically no attempts seem to have been made to study the survival of bacteria in naturally generated air-borne clouds; almost all of the useful work on survival of naturally dispersed organisms is based on a study of the bacteria in dust or in material contaminated with feces. Lidwell & Lowbury (72, 73) found that *Staphylococcus aureus*, *Streptococcus pyogenes*, and the general mixed flora in natural floor dust all showed an increased death rate with increasing humidity; they confirmed the lethal effect of sunlight and showed that even a fluorescent lamp had some killing effect. Gram-negative bacilli may survive better in moist than in dry conditions [Idina (63a)].

Heterogeneous as it is, the work on survival suggests that death outside the host is unlikely to be a factor that prevents the intramural spread of bacteria. To be a limiting factor for the organisms actually in the air, the death rate would have to be very rapid, since ventilation and deposition will ordinarily remove air-borne organisms after a short time. And the abundance of staphylococci and streptococci so often demonstrated in the dust in hospital wards, schools, and the like, show that, even if many die, yet many still survive, and one would think that the survivors are sufficiently numerous to serve as a source of infection.

For viruses other than smallpox, which can survive for months, we have practically no useful evidence, though general laboratory experience suggests that they survive much less well than bacteria.

It might be that bacteria survive outside the body, but lose virulence in the process. Early work denying this [Buchbinder, Solowey & Solotorovsky (19)] suffered from the fact that the dust bacteria were grown in laboratory media before being tested for virulence. We need to know whether, while still in the dust, they remain virulent. Rammelkamp and his colleagues (95) have recently suggested that they do not. These workers attempted to infect volunteers with floor dust containing large numbers of viable hemolytic streptococci, but failed; the volunteers could only be infected by administration of moist secretion. These experimental results were supported by previous epidemiological observations (see below) but they were based on very small numbers of volunteers, and are so far unsupported by any evidence from experimental animal infection. Nevertheless, the bacteria that survive in an aerosol may certainly be altered in their physiological characters, as shown for *Pasteurella pestis* by Berendt (9).

LABORATORY STUDIES OF AIR DISINFECTANTS

Reviews are available of much of the earlier work on aerial bactericides [Williams (121)] and comparatively little has been published recently. It will suffice to recall that physical and chemical methods have been used. In

practice, ultraviolet radiation (of 253.7 m μ wavelength) is the only physical method to have been tested extensively, and triethylene glycol, hexylresorcinol, α -hydroxy- α -methyl butyric acid, and sodium hypochlorite are the only chemical substances.

Laboratory tests of air disinfectants have practically always given unduly optimistic assessments of the probable value of a particular chemical for killing bacteria found in the air in normally occupied places. This has arisen partly from a tendency of laboratory workers to experiment with sprayed bacterial cultures of Gram-negative bacilli, which seems to offer a test system calculated to flatter even the poorest of disinfectants. Bourdillon *et al.* (16) recommended the use of natural saliva, with its contained streptococci, as a test cloud but it seems that even these are far more sensitive than the dust-borne bacteria that make up the bulk of the air flora; whether they are more resistant than the air-borne pathogens that one is trying to kill is not really known.

The difference in effectiveness of aerial bactericides in the laboratory and field may also stem from the method by which both the bacterial cloud and the bactericide are generated. Nash (86) stressed the importance of rate of vaporization of glycols in creating a volume of air containing a particularly high concentration of disinfectant, and also the value of minute droplets of glycol in the air as a reservoir for further vaporization. Darlow & Powell (26) considered that hexylresorcinol might be much more effective in killing bacteria in droplets that were in the process of drying rather than those already in the dried state.

Other disinfectant methods.—We have already stressed the importance of clothes and bedclothes in amplifying the dispersal of bacteria from infected persons; the neutralization of these reservoirs has naturally been studied extensively. Methods of causing fabrics to retain dust, as by their impregnation with oil, were tried first and were shown to be effective in reducing for example, the numbers of hemolytic streptococci dispersed into the air during bedmaking [see Wright, Cruickshank & Gunn (126)]. Recently, the emphasis has been more on disinfection of fabrics, by disinfectant rinses [Blowers & Wallace (13)], by disinfectant gases [Public Health Laboratory Service (94)], or by heat. Woolen blankets are not easy to launder at disinfectant temperatures and boilable cotton blankets have therefore been introduced as a substitute [Blowers, Potter & Wallace (12)]. So far, relatively few tests have been made of the efficiency of regular disinfection of the blankets in reducing aerial contamination with pathogenic bacteria.

Floors and other equipment that may act as secondary reservoirs of bacteria have also been studied similarly. Floors were treated with dust-laying oils from about 1941 onwards but, although demonstrably effective in reducing air-borne bacteria [van den Ende, Lush & Edward (113)], the practice has never been widely adopted, partly because of its aesthetic and practical disadvantages and partly because of its failure to influence the amount of cross-infection. Disinfectant preparations have been remarkably

little tested, but a recent report suggests that orthophenyl-phenol may prove to be useful [Dunklin & Lester (39)], and there are suggestive preliminary results from the use of organic tin compounds [Hudson, Sanger & Sproul (62)].

LABORATORY EXPERIMENTS

DISPERSAL OF MICROBES FROM HUMAN BEINGS

Respiratory tract.—Although many investigations were made of the dispersal of bacteria from the mouth and nose in the early years of the century, it was not until after Wells' (119) demonstration of the potential importance of the really small droplets that useful analysis became possible. The rate at which droplets of saliva, for example, settle toward the floor depends, of course, on their size. Wells pointed out that some of the droplets expelled from the mouth must be so small that their contained water would have evaporated before they could have time to reach the floor; the settling rate of the solid residues of such droplets (including any bacteria or viruses) would then be extremely low. Such residues were called "droplet nuclei." For example, a $100\text{-}\mu$ sphere of unit density falls two meters in still air in about 6 sec.; a $1\text{-}\mu$ sphere takes $16\frac{1}{2}$ hrs. to fall the same distance. In dry air, a $100\text{-}\mu$ droplet of pure water evaporates completely in 2 sec., i.e., in less time than it would take to fall from the mouth to the floor. The rate of evaporation of a salivary droplet is slower than that of a pure water droplet [see Lidwell (69)] but the principles are the same.

The existence of very small droplets in the spray from a cough or sneeze was demonstrated with high speed photography by Bourdillon & Lidwell (15), but the first good analysis of the bacteriological characteristics of the dispersed clouds was made by Duguid (34, 35, 36).

Sneezing produces by far the largest number of particles but the very great majority of them contain no bacteria even though they originate from the atomization of saliva containing 10^9 organisms per ml. Sneeze droplets seem to come mostly from the saliva in the anterior part of the mouth and contain predominantly the microbes present there. Coughing produces far fewer droplets, and, though these appear to originate from the pharynx, no very large number of infected particles are dispersed, even by throat carriers. The streptococcus-infected particles in a simulated sneeze were found by Lidwell, Noble & Dolphin (74) to contain an average of about six viable units, with a range from 11 for particles over 18μ in diameter to 2.4 for particles less than 4μ . The number of bacteria expelled from the mouth during reading aloud is, in part, related to the rate of secretion of saliva [Rubbo & Benjamin (105)]. Relatively few bacteria are dispersed from the nose, except in very vigorous "snorting" [see also Hare & Thomas (52); Shooter, Smith & Hunter (109)].

Nasal carriers of hemolytic streptococci produce far greater contamination of their environment than throat carriers; as they do not commonly sneeze, some mechanism other than direct expulsion of droplets must be

postulated, and this is generally held to be contact of the hands with the nose, and transfer of the bacteria on the hands to secondary reservoirs like handkerchiefs, clothing or bedclothes from which further dispersal may take place [Hamburger (49); Hare & Thomas (52)]. Indeed, gentle shaking of a handkerchief may liberate 15,000 bacteria-carrying particles into the air, with a large proportion of staphylococci in the case of nasal carriers [Dumbell *et al.* (38)].

Skin.—The skin may serve as a vehicle for spreading respiratory tract organisms directly, as in handshaking [Hamburger (49)], but it may also be a primary source of dispersal. The skin lesions of eczema and impetigo are often infected with staphylococci and streptococci, while staphylococci certainly multiply even on the healthy skin of some persons. Dispersal from the skin, if not by direct contact, must be by contamination of clothes, from which the bacteria may become air-borne. Carriage on the skin of the perineum may be particularly potent source of clothing contamination and air-borne dispersal [Hare & Ridley (51)].

Intestinal canal.—In hospital wards for infants and children with diarrheal diseases caused by *Salmonellae* or enteropathogenic *Escherichia coli* there is often widespread contamination of the ward equipment and floor dust [Rogers (100); Rubbo (104)]. The bacteria from the feces are probably disseminated largely from soiled napkins taken from the infants, though soiled bedclothes and nurses' uniforms must all, on drying, be potential sources of air-borne particles. Similar wide distribution of *Shigella sonnei* has been found in the lavatories of schools during an outbreak of dysentery [Hutchinson (63)] and there appears to be actual formation of an aerosol in the flushing of water closets [Darlow & Bale (25)]. Bacteria from the infected urinary tract presumably spread in the same way.

Secondary reservoirs.—At almost all times our clothing—whether dress or bedclothes—serve as a very efficient mechanism for dispersing the bacteria that we harbour. The nose or skin carrier of staphylococci who, naked, can distribute very few organisms by any activity, is converted into a most potent spreader by his clothes [Duguid & Wallace (37); Dumbell, Lovelock & Lowbury (38); Hare & Thomas (52)]. A similar effect has been shown for bedding on numerous occasions. In surgical wards wound dressings, and especially plaster casts when soaked with pus, allowed to dry and subsequently broken open, can be the source of considerable air-borne clouds as well as the source of contact contamination [Bourdillon & Colebrook (14); Girdlestone & Bourdillon (45)].

Apparently no one has yet determined the size distribution of the air-borne bacteria-carrying particles generated from infected clothing. The recent studies of Hare and his colleagues have all depended on settling plates for determining aerial dispersal, a method that gives undue prevalence to the particles too large to become air-borne, and which are, therefore, unlikely to be responsible for spread at a distance. The air-borne staphylococci in a hospital ward are probably mostly derived from bedclothes and curtains; in a recent study 33, 42, 19, and 6 per cent, respectively, of the staphy-

lococcus-containing particles were found in the four particle-size ranges >18 , 10 to 18, 4 to 10 and $<4 \mu$ [Lidwell *et al.* (74)]. The mean number of viable staphylococci per particle was about four.

Relative importance of various routes of dispersal.—Pathogenic microbes can, therefore, be dispersed from their host (a) by direct contact; (b) into the air directly from sites of infection in the respiratory tract either as large projectile droplets (giving rise to "direct droplet infection") or as small air-borne nuclei (responsible for "direct air-borne infection"); or (c) into the air indirectly from infected sites anywhere in the body through the mediation of clothes and the like (giving "indirect air-borne infection"). Very few attempts have been made to measure the relative numbers of pathogenic microbes dispersed in various ways, or indeed even to attempt any measure of the number liberated, though Duguid & Wallace (37) estimated that 10^6 bacteria may be liberated from each person's clothes each day. Lovelock (personal communication) made some ingenious experiments with fluorescent tracer materials, which suggested that a chain of physical contact could convey far more pathogens from the nose of one person to the face of another than could aerial spread. But in these experiments fluid was continually dripped into the nose and the opportunities for contact, in volunteers playing cards, were considerable.

INTRODUCTION OF MICROBES INTO THE NEW HOST

Respiratory tract.—The relative danger of different modes of dispersal of air-borne particles cannot be assessed simply in terms of the relative numbers of microbes dispersed by various routes, as becomes immediately apparent when inhalation infection is considered; it must also depend on the sizes of the particles distributed. In nose breathing practically all inhaled particles over about $5\text{-}\mu$ diameter are retained in the nasal passages; with mouth breathing some of these may reach the larger bronchi [Brown *et al.* (18)]. Nasal retention becomes progressively less with particles below 5μ in diameter, and $1\text{-}\mu$ particles practically all penetrate to and are in large part retained in the depths of the lungs, especially the alveoli. This general pattern has been established both on a theoretical basis, and by experiments with various inert dusts [Brown *et al.* (18); Davies (27)] and with radioactive particles [Harper & Morton (55)]. In small animals nasal retention is rather greater than in man but 1μ is still the particle diameter most effectively retained in the alveoli [Palm, McNerney & Hatch (88)]. Complications may be introduced when the inhaled particles are hygroscopic, because the air in the respiratory tract is almost saturated with water and such particles increase in size after inhalation. Particles reaching the terminal bronchioles and alveoli are likely to remain there for some time, but the higher regions of the bronchial tree are all lined with ciliated epithelium, and particles settling on this are carried up the bronchi and trachea to the larynx and eventually swallowed. There is some evidence that the initial distribution of inhaled microbes may be altered by alcohol, anesthesia, etc. [Ames & Nungester (3)].

As early as 1949 Sonkin (111) found differences in the dose of a group C

streptococcus needed to set up infection in mice according to the site of inoculation, in this case with a fluid suspension introduced by catheter. These results were later confirmed with air-borne pneumococci and group C streptococci sprayed from suspensions containing various concentrations of glycerol to vary the particle size [Sonkin (112)]. Different diseases resulted from infection in the upper and lower parts of the respiratory tract. The lung disease could be initiated by small doses of $2\text{-}\mu$ particles but needed 10^6 times as many organisms when $12\text{-}\mu$ particles were employed. The upper respiratory tract disease needed a larger dose than the lower, but the dose of organisms was only slightly affected by particle size. An elegant series of investigations have been carried out by Henderson and his co-workers [Druett *et al.* (31, 32, 33); Harper (53)], employing the "spinning top" aerosol generator devised by Walton & Prewitt (115), which produces a homogeneous cloud of particles of predetermined size, and a continuous flow aerosol apparatus from which experimental animals can inhale. For all of three infections, with *Bacillus anthracis*, *Pasteurella pestis*, and *Brucella suis*, the number of organisms needed to set up disease was greater by factors of 17, 2.5, and 600, for clouds with particle diameters of $12\text{ }\mu$ than with $1\text{-}\mu$ clouds. This was, in large part, attributable to the different susceptibilities of different areas of the respiratory tract, a difference especially marked with *B. suis*. With *P. pestis*, small particles produced a bronchopneumonia, the large, a more rapidly developing septicemia. The dose needed to produce infection by inhalation is generally greater than that required by intraperitoneal injection [Druett *et al.* (33)].

Other routes of introduction.—With one exception, no specific investigations seem to have been reported on any other method of introduction of pathogens into the new host. Direct settling must be potentially important for exposed wounds and in the contamination of exposed foods in infant nurseries; it also contributes to the pool of infected dust from which direct contact infection can occur.

The exception is a curious one: the conjunctiva. Moore (85) demonstrated unequivocally that the conjunctiva could be the portal of entry of *Salmonellae* in an epidemic among guinea pigs, and several workers have claimed that the conjunctiva is the natural portal of entry for the measles virus in children [see Banks (6); Papp (88a)] and have supported this with reports of prevention of spread of measles by the use of protective goggles or the conjunctival instillation of antiserum [Rusti (106)]. None of these reports has been supported by really critical controls, but they cannot be ignored. The physics of conjunctival entry needs some explanation. Settling of truly air-borne microbes on the conjunctiva must be exceedingly rare because of its vertical disposition, and small air-borne particles can very rarely be travelling fast enough to impinge. The infecting agents must reach the conjunctiva either in large projectile droplets or, secondarily, by contact with infected hands, etc.

Importance of particle size.—The size of the particles with which patho-

genic microbes are associated when they are disseminated from the infected person is thus of manifold relevance. Some actions, like sneezing, produce many small droplets probably coming predominantly from the front of the mouth; coughing generates fewer particles, but perhaps more from the pharynx. The small particles can be carried long distances on air currents and could conceivably infect at a distance both in space and time. But a small droplet can carry very few, perhaps no more than one, bacterium or virus particle. On the other hand, the small droplets can, on inhalation, penetrate to the alveoli, but are not so likely to be trapped in the nose. Animal experiments show that small particles may be very inefficient at setting up nasal infection; large particles are better but they can only travel short distances.

These considerations serve to highlight the enormous gaps in our knowledge of the relevant facts about the transmission of human disease, and the consequent difficulties in knowing how to extrapolate the work with experimental animal infections to natural disease in man. We know practically nothing of the size distribution of the particles carrying pathogenic bacteria or viruses and we can only guess at the probable frequency with which any infective particles are dispersed at all, from our knowledge which is largely qualitative, of the distribution of bacterial pathogens in infected people. Concerning the viruses, our knowledge is even scantier. And of the recipient we are almost equally ignorant. It seems reasonable to think that respiratory tuberculosis must arise from tubercle bacilli deposited in the alveoli in man, as in animals [Ratcliffe & Palladino (96)]. Perhaps the common cold virus may be best deposited in the nose, although, subjectively, colds often start with a sore throat or a tracheitis. Wannamaker (116) has suggested that the streptococci causing acute sore throat may first be trapped in the nose and subsequently transferred to the throat. We are ignorant, not only of the relative importance of different sites of infection, but also even of the order of magnitude of the dose needed to set up infection. So we cannot judge whether it is reasonable to think that droplet nuclei and dust particles that are small enough to be carried on air currents can set up infection, or whether the number of bacteria needed demands the deposition of many, or of large, particles.

The recent development of size-grading samplers, especially that described by Lidwell which is capable of sampling air at 20 cu.ft. per min., offers a possibility of answering some of these questions, but the difficulties are still formidable. The pathogens are rarely as easy to cultivate selectively as is the hemolytic streptococcus. And, in the ordinary way, their presence in the air is capricious so that special efforts will be needed to discover suitable circumstances for doing the sampling.

The interest in determining the relative importance of the different routes of spread is not merely academic; it is a prerequisite for intelligent application of specific hygienic measures for the prevention of disease in the same way as demonstration of the importance of water as a vector for ty-

phoid fever, or of mosquitoes for malaria, was essential to the control of those diseases. In the absence of methods for obtaining direct evidence on modes of transmission, we have recourse to epidemiological studies. If these are often difficult to interpret, they at least deal with the real disease in real-life circumstances and demand no doubtful extrapolation from laboratory experiments on small animals.

EPIDEMIOLOGICAL INVESTIGATIONS

The epidemiological method is at its most convincing in unraveling the spread of diseases in which many persons are infected from a common source, or where infections occur through contact with particular vectors and this contact can be decisively interrupted. None of these conditions holds for the bulk of the diseases we are considering here. When individuals are in a position to infect one another through small air-borne droplet nuclei, they are usually also able to transmit large droplets and to contaminate fabrics and floor dust to serve for indirect air-borne spread. And, although methods are available to attack these routes severally, it is rare that any method closes its particular route absolutely. Moreover, it has rarely been possible to conduct the experiments in a completely closed community, and too often the subjects have had opportunities for contracting infections outside the experimental environment. It is perhaps noteworthy that the most convincing report of clinical success with an air disinfectant was from a trial in a hospital ward with bedridden children [Harris & Stokes (57)].

During recent years there have been two main areas of discussion. For the diseases whose causal microbes are thought to enter through the respiratory tract—respiratory diseases and many of the systemic diseases—there has been conflict between the proponents of short-range direct-droplet infection on the one hand and air-borne infection on the other, with extensive secondary conflict over the relative importance of the direct (droplet-nucleus) and indirect air-borne (dust, etc.) routes. In the field of hospital cross-infection, in surgical wards, maternity nurseries, and, for the diarrheal diseases, in children's wards, the conflict has been between the advocates of contact infection on hands, instruments, and other equipment on the one side, and air-borne infection, especially by the indirect route, on the other.

Only the most sanguine can imagine that the transmission of infection for any disease is accomplished by any one route (though it could well be that some diseases are never transmitted by a particular route). The problem must therefore be to decide on the relative importance of various routes of transfer in particular circumstances. But, at present, we are one stage behind in our knowledge, and all we can profitably do in this section is to review the literature for evidence whether particular routes of infection are ever important. It will be most convenient to do this under the headings of the conflicts just mentioned.

Diseases with respiratory tract entry.—It was commonly noticed that measles and chickenpox were especially likely to spread in infectious disease

hospitals even when the patients were nursed in separate rooms [Harries (56)], suggesting that air-borne infection was important. On the other hand, scarlet fever patients nursed in separate rooms did not suffer cross-infection with new types of streptococcus to the same extent as those nursed in the open ward [Allison & Brown (2)], suggesting that for the streptococcus closer contact might be necessary—a suggestion supported by Dingle, Rammelkamp & Wannamaker (30) and our own work in a children's home [Holmes & Williams (60)].

Any increase in the ventilation rate of an occupied space decreases the numbers of air-borne bacteria in it, and may be expected therefore to reduce air-borne spread of infection. In the London trials of ultraviolet irradiation in schools, measurements were made of the air-change rate in the school-rooms, but careful analysis failed to reveal any noteworthy correlation with the incidence of any of the common diseases, though there was some suggestion of a small correlation with the common cold [Reid *et al.* (97)]. In an analysis of the seasonal variations in the incidence of measles and chickenpox in different parts of the United States, Mildred Wells (118) found that the winter peaks of these diseases were shorter in the Southern States, and suggested that this might reflect the shorter period for which indoor ventilation rates would be reduced to winter levels. While studying the common cold in clerical offices in London, we observed a sudden rise in incidence association with a sudden drop in the external temperature and a sudden rise in the indoor *Streptococcus salivarius* counts, and postulated that the temperature drop might have led to the increased incidence by stimulating a reduction in the ventilation rate [Reid, Williams & Hirsch (98)], but subsequent and more extensive experience, still in the course of analysis, does not appear to support this explanation.

In experiments with human volunteers [Lovelock *et al.* (78)] it seemed that the common cold could be transmitted by droplet nuclei or by close contact, including possibly direct droplet infection. Inhalation of the infective suspension was less effective than instillation, and no success was attained through attempts to transmit infection by dried handkerchiefs and the like, but the difficulties encountered in artificial transfer of the common cold are considerable so that no great weight can be put on these comparative tests.

Indirect air-borne spread has been postulated to explain the spread of *E. coli* diarrhea from one cubicle to another [Rogers (100)] but the possibility of contact infection could not be excluded.

Really distant air-borne spread has also been claimed for smallpox, even to the extent of spread extramurally from the hospital to its neighbourhood [Peirce *et al.* (89); Power (93)].

There seems to be more chance of disentangling the paths of spread by controlled experiment. Air disinfectants can be effective only against small air-borne particles, so that control of a disease by them would indicate that it was spread by the air-borne route. Disinfectants and the like applied to

floors and to the fabric reservoirs of bacteria and viruses could affect only diseases spread by the indirect air-borne route. The wearing of face masks could control diseases spread only by direct droplet infection, and not by direct air-borne infection since numerous small bacteria-carrying particles escape round the edges of most face masks [see Shooter, Smith & Hunter (109)].

Ultraviolet radiation is the air disinfectant method most extensively tested. Wells, Wells & Wilder (120) reported a long series of studies conducted in Pennsylvania schools in which the irradiation of the upper air reduced the epidemic spread of measles and probably also of chickenpox and mumps. Similar experiments made in several schools in a London suburb [Medical Research Council (84)] offered some confirmation of these results in that the secondary attack rate following the introduction of a case of measles was reduced from 19 to 13 per cent; neither mumps nor chickenpox had sufficiently wide distribution in the three years of the experiment to offer an opportunity for detecting an effect. A similarly small effect was also recorded by Bahlke, Silverman & Ingraham (5) from New York State. In neither the New York nor the London studies was the total attack rate for measles reduced by the irradiation, but this doubtless reflects merely the great susceptibility of young children to the disease. All the experiments concur in suggesting that the spread of measles is affected by upper air irradiation and that air-borne infection therefore probably plays some part. In the London studies there was a good correlation between the counts of *Streptococcus salivarius* in the schoolroom air and the secondary attack rate for measles [Reid, Lidwell & Williams (97)], which agrees with the idea that this bacterial count measures contamination of the air with droplet nuclei from the respiratory tract.

In the London studies and also in a similar but much shorter test in Milan [Castoldi (21)], there was a suggestion that scarlet fever and acute sore throat were reduced in frequency in the irradiated schools. It has been an almost general experience that "the common cold" is not affected by ultraviolet air disinfection.

Ultraviolet radiation was also employed in the form of "curtains" at the entrance to hospital cubicles in several studies in United States children's hospitals [Rosenstern (102)], and effective control of respiratory tract cross-infection was claimed. Unfortunately, these results were quoted in insufficient detail to make possible precise judgment as to what diseases were being controlled. Since 1941 considerable progress has been made in unraveling the etiology of some of the virus diseases and a critical re-examination of the value of ultraviolet curtains would be very useful. In view of the short time available for the irradiation to kill microbes carried on air currents through door-ways, the intensity must be about $250 \mu \text{ w./sq.cm.}$ at the centre of the aperture [Wedum, Hamel & Phillips (117)].

Although a variety of units have been designed to disinfect the air of a room by recirculating it over ultraviolet lamps [Harstad, Decker & Wedum

(58)], there is apparently no published evidence on their clinical efficacy. In principle, it must be remembered that even if the irradiation kills all the microbes in the circulating air, the rate of killing is limited by the volume of air recirculated, and a unit to give a significant air turnover has to be large; upper air irradiation of the whole room is usually a far more efficient method of employing the ultraviolet energy.

Bactericidal vapors seem to offer a more suitable air disinfectant because they may be all-pervading, rather than confined to the upper air of the room. In fact, this difference is more apparent than real, for it has been found that even with upper air irradiation there is sufficient reflection to provide a bactericidal intensity in the lower parts and this may contribute substantially to the disinfection [Medical Research Council (84)]. A serious limitation on the usefulness of chemical air disinfectants is their dependence on a particular level of humidity.

Only triethylene glycol has been submitted to substantial field trials. The results can be summed up very simply. In only one study was definite control of disease reported [Harris & Stokes (57)]: the common cold in a trial in a ward of bedridden children. In some studies an effect on air-borne bacteria has been observed without any effect on clinical disease [Loosli *et al.* (77)], and in some not even this has been seen [Krugman & Ward (67)]. One field trial of hexylresorcinol also failed to demonstrate any bactericidal or clinical effect [Lidwell & Williams (75)]. A test of our own with α -hydroxy- α -methyl butyric acid in clerical offices (to be published) demonstrated a bactericidal effect similar to that of ultraviolet irradiation, but no clinical effect.

From a practical point of view it is evident that no air disinfectant is at present useful. It seems that the best agent for use as a tool for distinguishing paths of spread of infection is ultraviolet irradiation, for with it substantial killing of air-borne microbes is demonstrable. That it fails to do more in controlling disease may be because the diseases studied are spread by routes that are not susceptible to air disinfectants, particularly the direct droplet route, or because the killing, although substantial, is incomplete and sufficient numbers of pathogens survive to infect [see Medical Research Council (84) for discussion of this point].

The ease with which floor dust and blankets in hospitals yield large numbers of hemolytic streptococci led to the idea that these things must be important vectors of infection, and stimulated a great deal of work on dust suppressive measures. It was easy to show that treatment of floors and blankets with oil emulsions reduced the numbers of streptococci dispersed into the air but, as with the air disinfectants, there are practically no reports of the successful control of disease, and several of failure to control it. In one instance, a really dramatic reduction of streptococcal secondary infection in a measles ward was recorded [Wright, Cruickshank & Gunn (126)] following oil treatment of floor and bedding; no one else has ever produced such an effect but probably no one else has tested the method in the face of an epi-

demic in which streptococci were spreading so vigorously. In extensive trials in U. S. Army installations where the streptococcal infection rates among normal men were 31 per 1000 per week, some suggestion of a reduction was obtained when dust control methods were used [Loosli *et al.* (76)]. Clearly, the situation of this test cannot really be compared with that of a measles ward in an old infectious diseases hospital, where one could hardly hope to observe a really dramatic effect.

These results would therefore suggest that, in some circumstances, streptococci may spread by the indirect air-borne route. However, the experiments of Rammelkamp and his colleagues [Perry *et al.* (91, 92); Rammelkamp *et al.* (95)] already referred to, suggest that streptococci dried on blankets and in floor dust were not likely to be infective. For obvious reasons Rammelkamp's volunteer experiments had to be on limited numbers and, without a knowledge of the dose that actually infects men in naturally occurring epidemics, his interpretation must be open to some doubt. But these observations do suggest caution in assuming that, because pathogenic bacteria can be found in a particular place, that place must be dangerous as a source of infection.

Nonrespiratory diseases.—In the field of wound cross-infection there is little doubt of the importance of "contact" routes of transfer, since several controlled trials of wound dressing routines specifically designed to protect against contact infection reduced the incidence of streptococcal cross-infection [Williams *et al.* (122)]. At the time of these studies, staphylococcal infection was less fashionable than streptococcal, but there was an indication in some that staphylococcal cross-infection was also reduced. No specific tests have been made of the prevention of contact infection with staphylococci, though it is generally assumed that the lessons of streptococcal infection are applicable. Certainly, persons who are themselves carriers of staphylococci can infect wounds, presumably by direct contact [McDonald & Timbury (81); Penikett, Knox & Liddell (90)].

The one situation in which air-borne infection of wounds seems to be established is in the operating theatre. The early work of Bourdillon indicated the risk of bacteria being sucked into operating rooms on air currents set up by the prevalent exhaust type of ventilation, and led to the adoption of positive pressure ventilation in the dressing room of the Burns Unit, Birmingham Accident Hospital [Bourdillon & Colebrook (14)]. But it remained for Blowers *et al.* (11) and Shooter *et al.* (110) to demonstrate that the adoption of positive pressure to replace exhaust ventilation of an operating room could reduce the incidence of wound infections [see also Lowbury (79)].

In surgical wards it has been shown that there is often a high concentration of the specific types of staphylococci during epidemics of cross-infection, and this suggested that air-borne infection was also potentially important in the wards and that physical isolation, in separate rooms, of patients with sepsis ought to reduce the spread of infection. Preliminary results confirmed this suggestion [Shooter *et al.* (110)].

Blankets, other bedclothing, and bedcurtains are certainly one, if not the principal source of air-borne staphylococci in hospital wards, and this has led to a great deal of interest in methods of neutralizing this source. Tests of oil impregnation in a surgical ward [Rountree (103); Clarke *et al.* (22)] revealed no clinical benefit; but oiling only reduces dispersal without killing the bacteria. Recently, more interest has been given to the disinfection of blankets, and there is one report that this may sometimes be useful [Caplan (20)]. Disinfection prevents transfer from one user of the blanket to the next, but none of the disinfectant processes seems able to make the blankets efficiently self-sterilizing. There is rapid recontamination of the blankets while they are in use so that periodic disinfection may have little total effect on the numbers of bacteria dispersed from them. Perhaps dust suppression is required as well as periodic disinfection.

In maternity nurseries it is also thought that contact transfer, on the hands of the attendants and on their clothes, is important but there is little direct evidence. Cook, Parrish & Shooter (23) found that babies became nasal carriers of staphylococci more slowly when they were handled only by nurses wearing sterile gowns kept separate for each baby; merely wearing a gown while in the nursery was not sufficient. There is no direct evidence that aerial transfer is important, although, as in surgical wards, the numbers of staphylococci found in the environment suggest that it ought to occur. Reduction of the load of infection, as by disinfectant treatment of the babies' umbilical stumps [Jellard (64)], seems to reduce the spread of staphylococci but clearly this would be likely in any hypothesis of the mode of spread. The fact that the umbilicus seems often to be colonized before the nose points to contact infection rather than inhalation, but air-borne staphylococci could be responsible if they infected by settling.

The spread of *Salmonella* and *E. coli* in epidemic diarrhea in children's wards is almost certainly largely by contact—on the hands of nurses who have to handle the infected babies and sometimes also the food of other babies—but air-borne spread has also been postulated because of the extent to which environmental contamination can be demonstrated [Rubbo (104); Rogers (100)]. No critical attempts to distinguish routes have been reported.

CONCLUSION

Textbooks of medicine commonly state now, as for years past, that the mode of spread of many diseases is by "droplet infection"; this term, in fact, probably implies only that the disease is not food- or insect-borne. The real progress of the last 20 years has been towards a clarification of our ideas on the ways in which microbes could be transmitted from person to person intramurally. What has not been attained is convincing evidence as to which of the various ways is most commonly employed by the pathogens causing natural human disease.

One of the obstacles to further progress has undoubtedly been an undue emphasis on bacteriology at the expense of epidemiology: the recurrent yield-

ing to the temptation to believe that, because bacteria can be found in some place, that place is necessarily on an important route in the transmission of disease; and, as a corollary, that removal of these bacteria must prevent the spread of infection. A more fundamental difficulty is that transmission experiments in animals, though productive of precise answers to laboratory questions, cannot be extrapolated to human disease; yet the tools so far developed for dissecting the transmission process in human communities have proved far too blunt. The real needs at present seem therefore to be to shake off the belief that what could conceivably happen necessarily does happen, and to develop new experimental methods for studying the transfer of infection in real life. These new methods must take account of the complexity of the epidemiological possibilities. It is very unlikely that any method of environmental control will have a really far reaching effect in reducing the total incidence of an infection, so that we shall need methods that detect small effects resulting from partial control of the spread of infection by only one of many pathways.

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PROTEIN SYNTHESIS IN MICROORGANISMS^{1,2}

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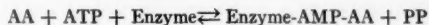
Protein synthesis is too large a subject to be treated comprehensively in the space allotted for this review. Fortunately, a number of more extensive reviews on the subject are available to readers who desire background material [Loftfield (1); Hoagland *et al.* (2); Hoagland (3); Chantrenne (4); Simkin (5); Roberts *et al.* (6); Gros (7); and Cohen & Gros (8)]. Induced enzyme formation (9), gene action (10), synchronous division (11), and enzyme localization (12) will be subjects of separate articles in this volume and will not be considered here. This review will be concerned with that area of protein synthesis of major interest to the author and will therefore be largely restricted to studies on the incorporation of amino acids into proteins and the synthesis of protein by cell-free preparations, with minor consideration of studies in whole cells and protoplasts. Attention will be focused on studies dealing with the sequence of enzymic steps between free amino acids and the formation of peptide bonds. In thus restricting the scope of this article it is inevitable that papers pertinent to the general subject will be overlooked or neglected; for any such omission I apologize now. Readability and more-critical appraisal of central points should benefit by such a treatment.

During the past six years the number of investigations concerned with the mechanism of protein synthesis in cell-free systems has been steadily increasing. As a result of this activity a generalized scheme has evolved that depicts, in a gross sense, the sequence of enzymic reactions that seem to describe the biochemical events leading to the synthesis of protein. Many of the observations leading to this scheme have come from studies, with mammalian preparations, pioneered by Zamecnik and Hoagland and their collaborators (1, 2, 3, 13), and contributed to by a number of other laboratories (14 to 19). Out of these collected observations has come the postulation of the following sequence.

Scheme 1

Sequence of Events Leading to Protein Synthesis

Stage 1. Activation of amino acids

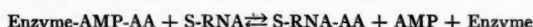


¹ The survey of the literature pertaining to this review was concluded in December, 1959.

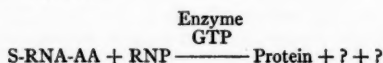
² The following abbreviations will be used, ATP (adenosine triphosphate); ADP (adenosine diphosphate); AMP (adenosine monophosphate); GTP (guanosine triphosphate); CTP (cytosine triphosphate); RNP (ribonucleoprotein); S-RNA (soluble RNA or transfer RNA); S-RNA-AA (soluble or transfer RNA charged with amino acids); PP (inorganic pyrophosphate); pCpCpA (5'-O-phosphoryl-cytidylyl-(3'-5')-cytidylyl-(3'-5')-adenosine).

³ Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

Stage 2. Transfer of activated amino acid to carrier RNA



Stage 3. Transfer to RNP, sequence determination, condensation, and release of protein



Some of these stages are better understood than others, and it is likely that stage 3 will eventually be broken up into three separate events.

Although this formulation may not accurately describe the actual events leading to protein synthesis, it nevertheless serves several useful purposes in giving us a framework within which to interpret results of further research and raises a number of questions that naturally suggest further experimentation. Some of those questions might be the following: (a) Is this a universal mechanism for the synthesis of protein used by all living cells or, as in many other biochemical sequences, are there alternate pathways? (b) Are all amino acids activated by the mechanism shown in stage 1, and does each amino acid have a separate and individual enzyme for its activation? (c) What are the nature, role, and specificity of S-RNA? (d) What are the structure and function of RNP? Is it template and if so how does it act? What is the role of GTP? And how many different steps occur in stage 3?

AMINO ACID ACTIVATION

In 1956 DeMoss & Novelli (20) observed that only about ten of the naturally occurring amino acids gave significant stimulation of PP-ATP exchange when added to soluble preparations from several bacteria. This raised the question of whether the remaining amino acids might be activated by a mechanism different from that of stage 1. In the meantime, several investigators observed the apparent activation of all of the naturally occurring amino acids by either PP-ATP exchange reaction or by the formation of amino acid hydroxamates [Nisman *et al.* (21); Cole *et al.* (22); Lipmann (15)]. Webster (23) reported that, although initially the activation of only seven amino acids could be observed, adjustment of pH and amino acid concentration revealed the activation of 18 amino acids and two amides in extracts of pig liver, pea seed, and yeast. Nisman (24) found enzymes for the activation of 18 amino acids in the low-speed pellet obtained by centrifugation of an osmotic lysate of penicillin protoplasts of *Escherichia coli*. Storage of these pellets at 0°C. for 24 hr. or sonic treatment for 10 min. severely depressed amino acid activation. In general, amino acids that had a weak activity in the fresh preparation were more severely damaged. D-Amino acids inhibited activation and a competition between L-valine, L-isoleucine, and L-leucine was observed. Nisman & Hirsch (25) subjected protoplasts of *E. coli* to osmotic lysis in the presence of 0.2 per cent digitonin and examined amino acid activation in the 30,000×g pellet, the 100,000×g pellet, and the resulting supernatant. Brief treatment (5 min.) with digitonin gave a 30,000×g

pellet that contained activating enzymes for all the amino acids, whereas longer treatment (45 min.) resulted in a loss from this pellet of activating enzymes for a number of amino acids. Little or no amino acid activation was found on the $100,000\times g$ pellet. In later notes, Nisman & Fukuhara (26, 27) report modifications of their procedures, giving preparations that do not activate amino acids but nevertheless can incorporate amino acids into protein. The membranes from osmotic lysis ($30,000\times g$ pellet) can be extracted at 0°C . with 50 per cent phenol. The phenol is removed and the extract precipitated with ethanol at -20°C . This fraction is said to incorporate amino acids into protein but to be devoid of activating enzymes. McCorquodale & Zillig (28) also examined the ability of various fractions from homogenates of *E. coli* to activate amino acids by PP-ATP exchange. Homogenates were prepared by shaking the organisms with glass beads in a vibrating homogenizer described by Zillig & Hölzel (29). They obtained four fractions, a "debris" fraction that sediments in 10 min. at $30,000\times g$, a ribonucleoprotein particle that sediments in 14 hr. at $105,000\times g$, a supernatant from the latter, and the protein electrophoretically isolated from the $100,000\times g$ supernatant. Activating enzymes for all 21 naturally occurring amino acids were reported to be present in the debris fraction, but in general these activities were low. Few, if any, activating enzymes were found on the nucleoprotein particle but most of them were present in the $100,000\times g$ supernatant and the protein isolated from it. The latter had less activity than the supernatant and may reflect losses during isolation. Gale (30, 31) reported that a membrane fraction derived from ultrasonically disrupted staphylococcal cells exhibited significant PP-ATP exchange with 11 of the amino acids tested. Glutamic acid is incorporated very well into protein by this preparation, but did not bring about the PP-ATP exchange. Hunter *et al.* (32) reported that membrane fractions isolated from osmotic lysates of *Bacillus megaterium* lysozyme protoplasts by centrifugation at $20,000\times g$ catalyzed a PP-ATP exchange at rates 100 to 300 per cent above control values with L-leucine, L-proline, L-asparagine, L-lysine, and L-histidine. It is stated that equivalent amounts of cytoplasm did not exhibit significant enzyme activity. When the membrane fraction was treated with ultrasound for 10 min. and centrifuged at $100,000\times g$ for 5 min., all of the activating enzymes for the above amino acids were found in the soluble portion and none in the residue. Spiegelman (33) likewise observed the presence of amino acid-activating enzymes in a $15,000\times g$ pellet prepared from osmotic lysates of *E. coli* that had been lightly ground with alumina and recentrifuged at $15,000\times g$. He states that these activities are retained through several washings of the pellet. It is noted, however, that the majority of the activating enzymes are present in the $100,000\times g$ supernatant. Hopkins (34) reports the activation of 12 amino acids by a pH 5.0 fraction derived from isolated calf thymus nuclei. D-Amino acids, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, and L-phenylalanine were inactive in catalyzing a PP-ATP exchange in this system. In the thymus cell the nuclei contain 70 per cent of the total amino acid activating ability, but with kidney cells the nucleus represents only 8 per cent

of the total activity. Baddiley *et al.* (35) found an enzyme in extracts of *Lactobacillus arabinosus* that catalyzes the PP-ATP exchange with D-alanine and not L-alanine and suggest that this enzyme may be concerned with cell wall synthesis.

A large discrepancy in the degree of activation of many amino acids, measured by PP-ATP exchange as compared with measurement of hydroxamate formation, was demonstrated (19) and the suggestion was made that the two methods might not measure the same reaction. Cormier & Novelli (36) found an enzyme in extracts of *Photobacterium fischeri* that catalyzed hydroxamate formation with glycine but not the PP-ATP exchange. Cormier *et al.* (37), using O¹⁸-labeled glycine, showed that the enzyme transfers one oxygen from the carboxyl group of glycine to the liberated orthophosphate, thus making it distinct from that of stage 1 that results in the transfer of oxygen to AMP (38). The purification of a threonine-activating enzyme that catalyzes the PP-ATP exchange, but not hydroxamate formation, was reported by Acs *et al.* (39). This finding confirms the suggestion that activation of amino acids leading to hydroxamate formation does not necessarily reflect the mechanism illustrated by stage 1. Webster & Davie (40) purified a serine-activating enzyme from beef pancreas that catalyzes the formation of serine hydroxamate and of ATP from adenylyl-serine and PP. The purified preparation showed variable PP-ATP exchange with serine. This seems to be the reverse of the situation with the threonine enzyme. Using hydroxamate formation as a measure of activation, Heller *et al.* (41) found that the pH 5.0 fraction of the silk glands of the silkworm activated tyrosine and tryptophan best and serine, alanine, and glycine poorly. Since the silk proteins contain 42 per cent glycine and 30 per cent serine, they suggested that incorporation of amino acids into these proteins must occur by means other than by carboxyl activation. Nohara & Ogata (42) observed that the formation of amino acid hydroxamates in the pH 5 fraction from liver was influenced by *p*-chloromercuribenzoate, monoiodoacetate, and azide, but that pretreatment of the enzyme with RNase markedly reduced hydroxamate formation, suggesting that hydroxylamine reacts with S-RNA-AA (stage 2) rather than with E-AMP-AA. A differential reactivity of these two intermediates with hydroxylamine as influenced by the particular amino acid, the activating enzyme, and the amount and kind of S-RNA present could account for the difference in activation as measured by the PP-ATP exchange or hydroxamate formation. A new and very sensitive method for measuring amino acid activation has been reported by Loftfield & Eigner (43). The method involves the use of radioactive substrates and selective chromatography on ion exchange paper. This method could prove useful for measuring many types of reactions, if the ion exchange papers become commercially available.

In amino acid activation we are left with three questions. First, the inability to demonstrate conclusively the activation of all amino acids by PP-ATP exchange in a given preparation. The report of the isolation of a threonine- (39) and a serine-activating (40) enzyme brings to eight the number of separate activating enzymes thus far purified. Enzymes for the activation of

the following amino acids have been described previously: tyryptophan (44), tyrosine (45, 46), leucine, valine, methionine (47, 48), and alanine (49). Thus, the number of specific activating enzymes continues to increase and it seems inevitable that enzymes for all the amino acids will eventually be reported.

A second question is the discrepancy between the ability of a preparation to incorporate a given amino acid into protein and to catalyze a PP-ATP exchange with the same amino acid. In microbial systems such discrepancies have been reported by Beljanski & Ochoa (50, 51), Gale (30), and by Nisman (27), and in mammalian tissues by Rendi & Hultin (52), Cohn (53), Fraser & Gutfreund (54), and by Fraser *et al.* (55). In a number of the cases cited, the suggestion has been made that the system contains a different amino acid-activating mechanism. This suggestion is made simply on the basis of an inability to find either PP-ATP exchange or amino acid hydroxamate formation in the presence of an amino acid that is actively incorporated. In no case, however, is positive evidence for a different activating mechanism presented. Gutfreund (56), with improved techniques for measuring amino acid activation, was unable to detect PP-ATP exchange with leucine or glycine; yet the preparations retained full ability to couple these amino acids with S-RNA (stage 2). Measurement of the relative rates of the two reactions (stages 1 and 2) led to the conclusion that stage 2 is rate limiting, and that low concentrations of E-AMP-AA that cannot be detected by the measurement of pyrophosphate exchange are sufficient to provide the observed rate of incorporation. This suggestion seems to be adequate explanation for the apparent exceptional systems and should be seriously considered until a positive finding of a different type of activation is reported.

The third question concerning amino acid activation is in the apparent location of the activating enzymes in two places in microbial extracts—on the cytoplasmic membrane fraction and in the soluble fraction. In mammalian systems, amino acid-activating enzymes are present exclusively in the soluble fraction and well-washed microsomes are devoid of activity. Similarly, in microorganisms, there are no activating enzymes on the ribosomes. The anomalous finding, however, is the considerable amount of activating enzymes sometimes found in the cytoplasmic membrane fraction. The activating enzymes can be liberated from the cytoplasmic membrane fraction by relatively mild procedures like brief sonic treatment, shaking with digitonin, or exposure to deoxycholate, although apparently not by simple washing. These observations suggest that the activating enzymes are not an integral part of the membrane fraction, but have been trapped in the fraction by coagulated bits of cytoplasm together with some of the particles from which they cannot be removed by washing, but require procedures that will break up the coagulum.

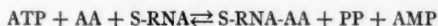
STAGE 2

Soluble ribonucleic acid.—Originally, it was impossible to show the accumulation of an aminoacyl-AMP as a free intermediate in amino acid activation and the reaction was written as involving an enzyme-bound inter-

mediate (20, 57). Chemical synthesis of leucyl-AMP and its enzymic conversion to ATP gave support to this reaction mechanism (58). Two greatly improved methods for the chemical synthesis of these compounds have now been described (59, 60). Nevertheless, the role of the aminoacyl adenylates as intermediates in the reaction remained conjectural until Karasek *et al.* (61) were able to isolate some enzymically formed tryptophanyl adenylate by using large amounts of tryptophan-activating enzyme. Kingdon *et al.* (62) showed that the amount of intermediate formed was stoichiometric with the amount of enzyme used. Thus, the enzyme-bound nature of the intermediate was established. The question of the acceptor remained a mystery until Holley (63) observed a ribonuclease-sensitive, alanine-dependent exchange of AMP with ATP. Then Hoagland *et al.* (64) and Ogata & Nohara (65) demonstrated that the activated amino acid is transferred to soluble RNA (S-RNA or transfer RNA) and was bound in an unstable linkage. The amino acid linkage to S-RNA is more stable than that of a carboxy-phosphoanhydride but more labile than an ordinary ester (64). The amino acid can be removed by treating the RNA with hydroxylamine at pH 8 to form the amino acid hydroxamate, or with dilute alkali to form the free amino acid. During studies with the purified tryptophan-activating enzyme, Lipmann's group (66a) noted the accumulation of the 2'/3' tryptophan ester of ATP and suggested that the amino acid linkage in RNA might be similar. Zachau *et al.* (66), by treating with ribonuclease S-RNA that had been pre-labeled with C¹⁴-leucine, isolated 2'/3' leucyl adenosine that was identified by chromatography with synthetic 2'/3' leucyl adenosine. The compound was characterized by its alkaline lability, giving rise to equal amounts of adenosine and amino acid, by reactivity with hydroxylaminé, and by its stability to periodate oxidation, suggesting esterification of one of the hydroxyl groups of adenosine. Berg (14) has similarly established that *E. coli*-activating enzymes transfer the activated amino acid to the terminal adenosine of S-RNA. Mild hydrolysis of *E. coli* S-RNA to give oligonucleotides (about ten nucleotides) gave inactive preparations. S-RNA is inactivated by snake venom phosphodiesterase when only 3 to 4 per cent hydrolysis is evident. Periodate treatment results in total loss of activity. Further treatment of this product with alkali followed by phosphomonoesterase to form a new end group fails to restore activity. If the latter treatment is carried out with S-RNA precharged with a specific amino acid, final treatment with alkali removes the amino acid and the S-RNA is active in accepting only the amino acid previously in position, not others, suggesting that an amino acid can protect its own specific sites. The amount of amino acids bound to RNA is a linear function of RNA (however, different amino acids are not bound to the same extent), but when different amino acids are incubated simultaneously the results are additive. Berg & Ofengand (14) have also established that the same enzyme that activates the amino acid also catalyzes the transfer to S-RNA by showing a constant ratio between activation and transfer of methione through a 100-fold purification of the methionine-activating enzyme from yeast. With the purified leucine-activating enzyme, the PP-ATP

exchange is 100 times as active as the transfer to S-RNA, indicating that the transfer to S-RNA is the rate-limiting step.

Preiss *et al.* (67) and Lipmann (16) measured the equilibrium constant of the reaction



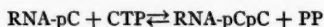
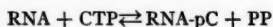
and found values of 0.32 and 0.7, respectively. These values indicate that a small free-energy change is involved in the formation of charged S-RNA and accounts for some of the properties of this amino acid ester linkage. Castell-franco *et al.* (68) had observed that synthetic aminoacyl adenylates labeled with C^{14} in the amino acid moiety could bring about the non-enzymic transfer of labeled amino acids to microsomal protein and also some non-enzymic incorporation of amino acid into RNA. The same group (69), however, have demonstrated a significant enzymic incorporation of tryptophan into RNA.

That transfer of amino acids to S-RNA was additive and that different amino acids did not compete with one another suggested either that there were different sites on RNA for different amino acids or each amino acid was transferred to a specific RNA. Schweet *et al.*'s (17) and Berg & Ofengand's (14) success in partially separating amino acid-specific RNA's implies that the latter is the case. Holley *et al.* (70) used countercurrent distribution to separate an alanine-specific RNA from a tyrosine RNA. Yields were low, however, and the increase in specific activity was only twofold. Schweet *et al.* (71) used chromatography over a Cato-8 cationic starch exchanger and elution with increasing concentrations of NaCl to purify a leucine-specific RNA about tenfold with about an 8 per cent recovery. Brown *et al.* (72) described an ingenious method for separating specific S-RNA's that might prove to be generally applicable. They took advantage of the fact that poly-diazostyrene at pH's between 7 and 8.5 formed insoluble complexes with tyrosine and histidine. Thus, S-RNA charged with either tyrosine or histidine could be separated from S-RNA molecules carrying other amino acids. The separated tyrosyl and histidyl S-RNA could be discharged with alkali. Recharging with only tyrosine and repeating the procedure allowed separation of the two species. The use of tyrosyl or histidyl peptides of other amino acids in this procedure should permit the separation of each of the specific S-RNA's.

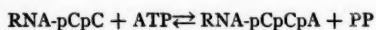
Many experiments suggest that there may be specific RNA molecules to react with each specific activating enzyme. The specificity is most certainly to be expected to reside in the base sequence, yet there does not seem to be significant differences in over-all base composition. One of the more interesting observations relates to the finding of additional, rare bases in RNA, such as pseudouridine (73 to 76), thymine (77), methylated adenines (77, 78), methylated guanines (78, 79), 5-methylcytosine (80, 81), and an additional sugar (82). Dunn (83) compared microsomal and S-RNA from rat liver for their content of these bases and found that S-RNA contains the following (in moles/100 moles of uridine): pseudouridine 25; 5-methylcytosine 10; 6-methylaminopurine 8.1; 6-dimethylaminopurine 0.1; 1-methylguanine 3.3;

2-methylamino-6-hydroxypurine 2.3; and 2-dimethylamino-6-hydroxypurine 3.0. Except for 6-dimethylaminopurine, all other bases are much higher in S-RNA than in microsomal RNA. Because of the relatively large amount of pseudouridine (5-ribosyluridine) in S-RNA, attention has been focused on it. Otaka *et al.* (84) found that in yeast cells the 80S particles containing 69 per cent of the total RNA had only a trace of pseudouridine but S-RNA, which was only 14.3 per cent of the total RNA, contained 14 moles of pseudouridine/100 moles of adenylic acid. Osawa & Otaka (85) found a striking correlation between the ability of S-RNA from yeast to accept leucine- C^{14} and its content of pseudouridine. The significance and function of pseudouridine and other minor components remains to be established, but their presence in the transfer RNA must be related to the amino acid specificity of the RNA and may also be involved in the coding problem. Koningsberger *et al.* (86) were the first to observe the presence of nucleotide-bound carboxyl activated amino acids in yeast RNA. Now an increasing number of publications have reported similar findings of nucleotide-bound peptides from RNA isolated from various bacteria, yeast, tumor cells, *Chlorella*, and a variety of mammalian tissues (87 to 93). Such compounds may play a role in protein synthesis, although no evidence has been presented to support this speculation.

Hecht *et al.* (94) have extended their previous studies on the sequence of terminal nucleotides in S-RNA (95) and have established that S-RNA must have a terminal adenosine moiety to accept amino acids. Freshly isolated S-RNA from rat liver pH 5.0 fraction generally contains pCpCpA terminal trinucleotide configuration and is at least partially charged with amino acids. (In this respect the S-RNA isolated from *E. coli* is said to be free of amino acids, the difference can perhaps be explained on the assumption that amino acids are lost during isolation of S-RNA from *E. coli*.) Incubation of the pH 5.0 fraction in the absence of CTP, ATP, and amino acids and in the presence of PP and AMP results in a loss of the three terminal nucleotides from the S-RNA. Use of S-RNA from which the end groups have been removed in this manner permits three separate steps to be defined. First, two cytosine nucleotide end groups are added with the elimination of PP.



Next, an adenine nucleotide is added when RNA-pCpC is incubated with ATP at a concentration that is insufficient for activation and transfer of amino acids to S-RNA.



Finally, by a combination of stages 1 and 2, the amino acids are activated and transferred to the reconstituted RNA. The enzyme adding the nucleotide end groups is the same for CTP and ATP but different from the enzyme that activates the amino acids. A calculation on the basis of the number of adenine end groups added shows one nucleotide incorporated per 98 nucleotide residues, giving a molecular weight of 33,000 (94). Preiss *et al.* (67) re-

ported a molecular weight for *E. coli* S-RNA between 30,000 and 50,000 and confirm that the terminal nucleotide is adenosine. Watson (96) found that S-RNA isolated from log phase cells of *E. coli* has a sedimentation constant of 3.95, giving a molecular weight of $25,000 \pm 2000$ or about 80 nucleotides. By alkaline hydrolysis, adenosine was found as the only terminal nucleotide and there was one adenosine per 84 nucleotides, thus confirming the molecular weight. Watson also found 2 moles of pseudouridine/mole of S-RNA, and approximately 1 mole of thymine/mole S-RNA. *Bacillus megaterium* S-RNA on the other hand did not contain thymine. *E. coli* S-RNA gave the same sedimentation constant when determined in the $100,000 \times g$ supernatant as when purified through phenol extraction. From this it can be inferred that S-RNA in *E. coli* exists free, and not combined with protein. A similar analysis of rat liver S-RNA by Bloemendal & Bosch (97) indicated that in the native state, soluble RNA occurs as a ribonucleoprotein that precipitates and dissociates from protein at pH 5.2.

Although there seems to be a specific S-RNA for each amino acid, the question of the species specificity of S-RNA is somewhat obscure. Berg & Ofengand (14) reported that RNA prepared from yeast, *Azotobacter vinelandii*, turnip yellow mosaic virus, tobacco mosaic virus, and rat liver had less than 5 per cent of the activity of S-RNA from *E. coli* when tested with a valine-activating enzyme from *E. coli*. On the other hand, Schweet *et al.* (17) reported that S-RNA from rat liver was as active as that from ascites cells with the pH 5.0 fraction from ascites cells. However, Webster (98) has recently described the purification of two apparently different alanine-activating enzymes from pig liver. One, derived from the cytoplasm and purified 3800-fold, is active in transferring alanine only to RNA derived either from pig liver cytoplasm or pig muscle cytoplasm. The other enzyme, derived from pig liver nuclei, was purified 30-fold and it transferred alanine best to RNA derived from pig liver nuclei. With both activating enzymes, RNA from calf liver, yeast, or pea seed was essentially inactive. These results suggest that S-RNA not only exhibits species-specificity but that there are differences in a given organ between nuclear and cytoplasmic RNA for the transfer of the same amino acid. In contrast, Holley & Goldstein (99) purified an alanine-activating enzyme from rat liver 500-fold and reported that RNA prepared from *E. coli* was active with their enzymes. Berg (48) reported that a purified enzyme from *E. coli* that can activate both valine and isoleucine can transfer only the latter to RNA. A methionine-activating enzyme from *E. coli* can transfer three times as much methionine to *E. coli* RNA as a purified methionine-activating enzyme from yeast. Evidently, the *E. coli* enzyme can find more methionine sites on *E. coli* RNA than can the yeast enzyme.

STAGE 3

Studies of the kinetics of *in vivo* and *in vitro* incorporation of amino acids into various fractions from mammalian cells have established that microsomes are the major site of synthesis of soluble protein (100, 101). Although

mitochondria are capable of independent protein synthesis (102), it is the microsomes (or ribonucleoprotein particles of size 80S) that became labeled early and lose their label as the soluble protein increases in radioactivity when an animal (103) or cells (104) are exposed to tracer amounts of labeled amino acids. Thus, it is generally believed that the RNP contains templates for the assembly of amino acids into specific proteins. After assembly, the labeled protein is believed to come off the RNP and become associated with the cytoplasm. The most striking difference between mammalian and microbial cells is that the important site of protein synthesis in microbial cells seems to be the cytoplasmic membrane. Spiegelman (33) with *E. coli* protoplasts, Gale (30) with disrupted staphylococcal cells, Butler *et al.* (105) with *B. megaterium*, and Connel *et al.* (106) with *Azotobacter* have all reported that the most extensive incorporation of amino acids into protein occurs in a fraction sedimenting at relatively low gravitational force and consisting largely of cytoplasmic membranes. When the various fractions are tested for *in vitro* incorporation the same pattern emerges.

Gale (30, 31) has published detailed summaries of the work emanating from his laboratory during the past several years on the incorporation of amino acids into protein by disrupted staphylococcal cells [see also (6)]. The points pertinent to the present discussion are as follows: (a) Incorporation takes place in a subcellular fraction that contains cytoplasmic membranes. (b) Two types of incorporation are recognized. Use of a single labeled amino acid (glutamic) gives a reversible incorporation. Electrophoresis on starch revealed that the radioactive glutamic acid is largely associated with a fast-moving component. This fraction called glutamyl-X is not free since treatment with ninhydrin did not liberate CO_2 ; it is highly unstable; i.e., 90 per cent of the glutamic acid can be liberated by treatment with dilute alkali. Only 50 per cent of the liberated glutamic is the L isomer. Some of the properties are similar to amino acid-charged S-RNA, but glutamyl-X differs from S-RNA in being soluble in trichloroacetic acid, perchloric acid, and 69 per cent ethanol. Reincubation with 18 amino acids leads to irreversible incorporation, and electrophoretic analysis shows that 50 per cent of glutamyl-X has been converted to a slow-moving component. (c) Incorporation by this preparation can be eliminated by pretreatment with ribonuclease, deoxyribonuclease, or extraction with 1.0 M NaCl. Incorporating ability is restored by adding nucleic acid or digests of nucleic acid. Fractionation of such digests by extraction with ethanol, paper electrophoresis, and paper chromatography yields "incorporation factors." This factor, or factors, apparently not nucleotide in nature, stimulates incorporation of glutamic acid into cell wall material, glutamyl-X, and the slower moving component. It does not however, affect the activation of amino acids. At the moment it is difficult to associate this stimulatory factor with any comparable fraction in mammalian systems.

Spiegelman (33) has reported the results of his investigations with osmotic

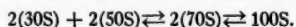
lysates of *E. coli* protoplasts [see also (6)]. Again, it should be emphasized that, both *in vivo* and *in vitro* experiments with this *E. coli* system, the low-speed fraction incorporates amino acids faster and to a greater extent than any other fraction. Hunter *et al.* (32) have extended their previous investigations of the incorporation of amino acids by the isolated cytoplasmic membranes of *B. megaterium*. By acid hydrolysis of membranes incubated with labeled amino acids it was shown that the incorporated amino acid is in peptide linkage. RNA isolated from the membrane fraction was labeled with amino acids in alkali labile linkage. Binding of C^{14} -amino acids to cytoplasmic RNA in tracer experiments with protoplasts was not observed although the RNA from the membrane fraction in the same experiment was labeled. Treatment of labeled membranes with sodium deoxycholate led to solubilization of 10 per cent of the protein. The specific activity of the lipoprotein fraction varied in amounts to five times as great as the residual protein. Extraction of membranes with boiling methanol removes 60 per cent of the labeled amino acid, and after such treatment the label is no longer TCA precipitable. Preliminary experiments indicate that the labeled amino acid is associated with the phospholipid fraction; and, when such labeling experiments are followed by further incubation with C^{12} amino acids, a quantitative transfer of label from the phospholipid fraction into protein is observed. The labeling of the phospholipid fraction is inhibited by chloramphenicol. The apparent involvement of a lipid fraction in the incorporation of amino acids is reminiscent of the report by Hendler (107) that intact cell preparations from hen oviduct accumulate radioactive alanine, phenylalanine, and valine into a lipid fraction that behaves kinetically like a precursor of protein. Connel *et al.* (106) have fractionated particles from *A. vinelandii* cells that were broken with sonic oscillation. Amino acid incorporation both *in vivo* and *in vitro* was much greater in a fraction sedimenting with the cell membrane. The 38S and 25S particles had little ability to incorporate amino acids. Schachtschabel & Zillig (108) have broken cells of *E. coli* by shaking them with glass beads in a special vibrating instrument. By differential centrifugation, three fractions are obtained: (a) a "debris fraction" sedimenting at $30,000\times g$; (b) a nucleoprotein fraction, sedimenting in 14 hr. at $100,000\times g$; and (c) the resulting supernatant. Both the debris fraction and the RNP particle (mostly 30S and 50S) incorporate C^{14} -amino acids into protein. Unlike the previously discussed systems, both particle fractions require supplementation with a pH 4.4 fraction from the supernatant, ATP, and S-RNA for full incorporating activity and are sensitive to ribonuclease. The debris fraction reaches a specific activity that is four times that of the RNP particle system. In contrast to the corresponding mammalian system,[†] these two systems from *E. coli* are not stimulated by GTP.

The transfer of amino acid from S-RNA to microsomes first observed by Hoagland (64) has now been shown to require an enzyme fraction from liver supernatant (109). This system has been fractionated by Grossi & Moldave

(110) into a dialyzable and non-dialyzable component, both of which are required for transfer. In contrast, Hunter *et al.* (32) were unable to obtain evidence that amino acid bound to RNA in the membrane fraction of *B. megaterium* could transfer the amino acid to protein. Tonomura & Novelli (111), using an *E. coli* preparation, observed the transfer of amino acids from S-RNA in a particle sedimenting at $24,000\times g$ corresponding to the membrane fraction, whereas the dialyzed supernatant (presumably containing 30S, 50S, and 70S particles) was a very poor acceptor of the label.

The inhibition of protein synthesis by chloramphenicol has been pinpointed by Lacks & Gros (112) at the transfer of S-RNA-AA to protein by showing that, when cells of *E. coli* are inhibited by chloramphenicol, the S-RNA becomes charged with radioactive amino acid without incorporation of amino acid into protein. Hopkins (34) made a similar observation when studying chloramphenicol inhibition of protein synthesis in calf thymus nuclei, an inhibition previously recorded by Breitman & Webster (113). Working with the $100,000\times g$ supernatant from streptomycin-sensitive and -resistant cells of *Mycobacterium friburgensis*, Erdös & Ullman (114) observed that dihydrostreptomycin inhibited the incorporation of C^{14} -tyrosine into protein in the extract prepared from the sensitive strain, but not in the extract from the resistant strain. Under the same conditions, streptomycin stimulated incorporation of C^{14} -tyrosine into S-RNA in both extracts. Erdös & Ullman concluded that streptomycin inhibits the transfer of amino acids from RNA to protein in the sensitive strain. Yarmolinsky & De La Haba (115) reported that puromycin inhibited the incorporation of amino acids in rat liver homogenates. Here, again, the inhibitory action of the antibiotic seems to occur at stage 3, in which the amino acid is transferred to protein. It is curious that three rather different antibiotics seem to inhibit protein synthesis at the same point. However, the transfer of amino acids from S-RNA to protein is a complex process that undoubtedly has multiple steps, and it is possible that these antibiotics are operating at different levels in stage 3. An alternative interpretation is that each antibiotic inhibits the activation of a different amino acid, thereby preventing completion of protein synthesis and resulting in a pile-up at the S-RNA stage. It should be mentioned that chloramphenicol has no effect on activation when a number of amino acids are tested simultaneously (20).

Recently, studies of ribonucleoprotein particles from bacterial cells have been emphasized (116). The size particle that can be isolated from cells seems to be strongly influenced by the ionic environment. Thus, Tissières & Watson (117) find that, at high Mg^{++} concentration (0.01 M), the particles from *E. coli* are largely 100S. As the Mg^{++} concentration is lowered, these will dissociate according to the following reaction (96):



The reversible transition between 100S and 70S occurs between 2×10^{-3} and 2×10^{-2} M Mg^{++} . The splitting of the 70S particle to a 30S and a 50S particle

occurs at Mg^{++} concentration of $2 \times 10^{-3} M$. If the Mg^{++} concentration is lowered to less than $2 \times 10^{-4} M$, an irreversible breakup of the particles to fragments of much lower sedimentation values results. The molecular weights for these particles are: 30S, 0.85×10^6 ; 50S, 1.8×10^6 ; 70S, 2.7×10^6 ; and 100S, 5.4×10^6 . The particles contain 63 per cent RNA and 37 per cent protein. The protein has two end groups of methionine and alanine, suggesting two peptide chains of molecular weight 25,000. Elson (118, 119) has reported, as has Bolton (120), the presence of latent RNase on these particles that remain inactive as long as the integrity of the particles is maintained. Elson has also observed the presence of DNase, and Bolton (120) found an amino peptidase present on the particles. In contrast to the RNase, the amino peptidase is reported to be active without disruption of the particle. The roles of these particles and of their constituent enzymes in protein synthesis remains to be determined. By kinetic analysis of the incorporation of amino acids into the soluble proteins and ribonucleoprotein particles of *E. coli* after brief (4 min.) exposure to isotope, the specific activity of the protein of the particles was lower than that of the soluble protein; it was concluded that the structural protein of the particles could not be precursor to soluble protein (116). A similar analysis of particle and soluble protein of yeast cells by Osawa & Hotta (121) gave similar results but no evidence for a flow of radioactivity between the two fractions. More recently, McQuillen *et al.* (122) reduced the time course of kinetic experiments to a few seconds and used S^{35} as a tracer to emphasize soluble protein synthesis (ribosomes are low in sulfur amino acids). With *E. coli* growing exponentially, a labeling to 5 sec. with S^{35} , followed by a "chaser" of cold sulfur, has revealed that the 70S ribosomes become saturated with nascent protein in 5 sec. and, in another 5 sec., this protein becomes associated with the soluble protein. The slower growth of particles, the smaller 30S and 50S ribosomes being made first and combining to form the 70S particles together with the brief exposure to isotope, has obviated the former difficulty of the labeling of the structural protein. Thus, it seems that the 70S particles are the sites on which soluble protein is made, and a calculation indicates that *E. coli* requires 5 sec. to make a polypeptide of 20,000 mol. wt. When cells were lysed with lysozyme followed by freezing and thawing, about half the ribosomes were released, and most of the remainder were detached from the residual membranous material by passing them through a pressure cell. The first fraction of ribosomes had only about half the specific radioactivity of the second. McQuillen *et al.* suggest that perhaps some of the particles exist free in the cell juice and others are more firmly bound to membranes and are more directly concerned in protein synthesis. This finding may explain the greater incorporating ability of the membrane fraction previously discussed. Mendelsohn & Tissières (123) studied the variation in the amount of ribonucleoprotein particles in *E. coli* as a function of the growth phase. They observed an increase in ribonucleoprotein particles that is paralleled by an increase in total RNA as cells passed from stationary phase through the lag phase and into

the logarithmic phase. This increase in ribonucleoprotein particles in the most active phase of growth is consistent with the suggestion that these particles are associated with protein synthesis.

OTHER INCORPORATING SYSTEMS

A number of reports describe systems that incorporate amino acids into protein and that apparently do not conform to scheme 1. Beljanski & Ochoa (50, 51) described a system from *Alcaligenes faecalis* that incorporates amino acids into protein, apparently by a different mechanism. The system consists of a low-speed particle (presumably cell walls and cytoplasmic membranes) and an enzyme ("incorporating enzyme") purified 100-fold from the supernatant and free of activating enzymes and S-RNA. The incorporating enzyme can replace the pH 5 fraction from liver in catalyzing the incorporation of amino acids into liver microsomes. Beljanski (124) reports the presence, in the highly purified "incorporation enzyme," of four specific enzymes that catalyze the exchange between nucleoside diphosphates and the homologous nucleoside triphosphates, and suggests that these exchange reactions may be associated with a different type of amino acid activation. It should be mentioned, however, that the exchange reactions are independent of added amino acids, and that the relation between these exchanges and the ability of the incorporating enzyme to catalyze amino acid incorporation has not been established. Another microbial system that seems to differ from the conventional scheme is reported by Nisman & Fukuhara (125, 126). Particles were prepared by differential centrifugation of *E. coli* protoplasts lysed with 5 per cent digitonin in 0.5 M sucrose. The particle sedimenting at $30,000\times g$ can incorporate radioactive methionine in the absence of the methionine-activating enzyme. The particle sedimenting at $105,000\times g$ that has little amino acid-activating enzymes brings about a lesser incorporation. The $30,000\times g$ particle catalyzes the incorporation of amino acids into a form extractable with hot trichloroacetic acid (S-RNA ?) and into protein. Both types of incorporation are stimulated by ATP, UTP, CTP, and GTP, Mn^{++} , the "equilibrium mixture" of amino acids, and by S-RNA as well as RNA prepared from the $30,000\times g$ particle or the $105,000\times g$ particle, with phenol. Both particulate preparations are reported to synthesize β -galactosidase when supplemented with the above components. Since the $30,000\times g$ particle incorporates methionine in the absence of the corresponding activating enzyme and yet the incorporation is stimulated by added S-RNA, the system is somewhat similar to the behavior of S-protein from liver reported by Rendi & Hultin (52) in that the S-protein apparently can transfer amino acids to S-RNA in the absence of amino acid-activating enzymes. Cohn (53) treated rat liver microsomes with lubrol and perfluorooctanoate and obtained a preparation that incorporated amino acids without the pH 5 fraction. The perfluorooctanoate pellet is reported to be free of activating enzymes. Rendi & Hultin (127) compared amino acid activation and incorporation in liver microsomes treated with lubrol or with lubrol and KCl. Lubrol treatment of microsomes increased the amino acid-activating ability,

presumably by making the indigenous amino acid-activating enzymes more accessible to added amino acids. Treatment of microsomes with lubrol in KCl removed the activating enzymes, which were found in the supernatant. Such treated microsomes could incorporate amino acids when supplemented with S-protein that is free of activating enzymes and S-RNA, suggesting that this preparation incorporates amino acids by a process not involving the usual activation and transfer to S-RNA.

GENERAL COMMENTS

Although there is no evidence now available that unequivocally establishes the fact that the events formulated in scheme 1 represent the mechanism of protein synthesis, the increasing mass of circumstantial evidence accumulating makes it highly likely that this reaction mechanism or one very nearly like it will be correct. In this particular scheme a number of important details remain to be worked out. These concern the structure and basis of the specificity of S-RNA, the role of GTP in the transfer of the activated amino acid from S-RNA, the organization of the activated amino acids in a specific sequence, their condensation and the final folding into the polypeptide structure. In view of the large research effort in this field, we can look forward to having answers to these questions in the near future. Granting that proteins may be synthesized through the reactions in scheme 1, we can ask whether this is the only mechanism by which proteins are made by all cells or are there alternate pathways. Recent investigations, already considered in this review, have suggested that alternate pathways may exist. The evidence upon which such claims are made falls into one category. Various subcellular fractions have been prepared that incorporate radioactive amino acids into protein, and in which either the activation of that amino acid cannot be demonstrated or in which the presence of S-RNA is in doubt. From these negative findings, the conclusion is reached that a different mechanism must therefore be operative. In view of the difficulties in measuring amino acid activation (i.e., fluoride inhibition in some cases and not in others), the deleterious effect of improper mixtures or concentrations of amino acids, for example, a negative finding, needs to be exhaustively investigated before being completely acceptable. In dealing with C^{14} -amino acids in subcellular systems we must always be aware that the specific activities measured in protein may appear large on a sensitive counter (especially if we add 10^6 counts) and yet represent a vanishingly minute reaction. But amino acid activation is measured, in micromoles or millimicromoles, and we do not yet know how small an activation reaction is required to yield the observed incorporation rates. Finally, it should be emphasized that to postulate successfully a different mechanism from that of scheme 1, it is necessary to demonstrate by independent criteria that a new or different reaction is indeed operating. I might add that all efforts in our own laboratory to find a different mechanism of protein synthesis with O^{18} as a tracer have been entirely unsuccessful.

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ENERGY METABOLISM IN CHEMOLITHOTROPIC BACTERIA^{1,2}

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INTRODUCTION

Until fairly recently, interest in the biochemistry of the chemolithotropic bacteria was confined to a small, but stout-hearted band of devotees who continued to struggle with these recalcitrant organisms despite the lures offered by the biochemistry of (say) the more cooperative *Escherichia coli*. Interest in the chemolithotropic bacteria has, however, been spreading; indeed, spreading to such an extent that the energy metabolism of these organisms now merits, and here receives, a full-length review. In it I have tried to restrict myself to recent work; those wishing to fill in the background should consult van Niel's review of autotrophic bacteria in general (1), the Symposium (2), or the monograph (3) on the same subject. The exergonic primary oxidations of the chemolithotrophs will be dealt with genus by genus since it is in this area of metabolism that the genera differ from one another; the endergonic reduction of carbon dioxide will be dealt with in a single section subsuming all genera since in this area of metabolism every autotroph behaves, in all probability, very much as every other autotroph.

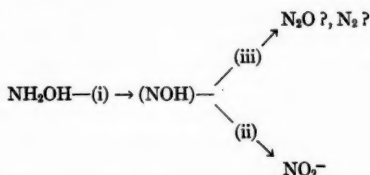
NITROSOMONAS

Six years have now passed since the first experimental evidence was obtained (4) in support of the suggestion made by Kluyver & Donker that hydroxylamine formation represented the first stage in the oxidation of ammonia by *Nitrosomonas*. Since that time, Imshenetskii and his school (5 to 10), working with autolysates of the organism have also found that these preparations are capable of oxidizing ammonia and hydroxylamine to nitrite. It is a little surprising that techniques involving autolysis are successful with *Nitrosomonas* since it is general experience that nitrifying organisms persist for long periods without substrate (11) and, therefore, by inference, do not readily autolyse. However, it may well be that the Russian strain has an unusually marked tendency towards autolysis, just as its ammonia-oxidizing systems have an unusually marked heat-stability (9). Engel & Alexander (12) have confirmed that intact cells of *Nitrosomonas* oxidize hydroxylamine to nitrite with the appropriate concomitant oxygen uptake. Their strain of the organism proved much less sensitive to higher concentrations of hydroxylamine than did the Aberdeen strain used by Lees (13); moreover, in contradistinction to the Aberdeen strain (14), its free-energy

¹ The survey of the literature pertaining to this review was concluded in October, 1959.

² The following abbreviations are used in the text: ~P for high energy phosphate bond; 2,4-DNP for 2,4-dinitrophenol.

efficiency did not fall as growth proceeded but remained constant at about 8 per cent over the whole growth period (15). Engel & Alexander also made the important observation that, during aging, *Nitrosomonas* lost its ammonia-oxidizing ability more rapidly than its hydroxylamine-oxidizing ability; this has been confirmed by my colleague Dr. J. H. Anderson. It has been argued (3, 16) that if H. Engel were correct in his supposition that ammonia is oxidized on the surface of *Nitrosomonas* cells, then this oxidation could be looked on as a device to convert the charged ammonium ion, which cannot penetrate the cell wall, into an uncharged hydroxylamine molecule that can. The loss of ammonia-oxidizing ability during aging may thus represent some slight modification of the structure of the cell wall with consequent impairment of the ammonia-oxidizing systems in it, the hydroxylamine-oxidizing systems within the cell meanwhile remaining intact. The intermediate that must exist between hydroxylamine and nitrite remains unidentified; apparently it is not hyponitrite since cell-free extracts that oxidize hydroxylamine will not oxidize hyponitrite (17). Under anaerobic conditions such extracts will decolorize methylene blue in the presence of hydroxylamine (15, 17); there is an output of gas but no nitrite is produced (17). Under aerobic conditions, methylene blue increased the amount of nitrite produced from hydroxylamine by the extracts, but the oxygen uptake observed during the oxidation did not correspond to the amount of nitrite produced. It seems that these and other results obtained by Anderson may be explicable on the basis of some such scheme as the following in which (NOH) represents the unidentified and unstable intermediate:



These are the reactions observed in the cell-free extracts: reaction (i) can take place with methylene blue as hydrogen acceptor, reaction (ii) apparently requires atmospheric oxygen for its completion and is inhibited by cyanide, while reaction (iii) is a consequence of the instability of the (NOH) [See reference (16) for a discussion on the nature of the (NOH)]. In whole cells, reaction (ii) presumably takes place so rapidly that loss of nitrogen from the system via reaction (iii) is negligible. Ammonia is not oxidized by these cell-free extracts and Anderson has made the interesting suggestion (18) that since the oxidation of ammonia to hydroxylamine is actually an endergonic process ($\Delta F = +4.7$ kcals.), ammonia activation (19) by $\sim\text{P}$ may be necessary for the oxidation to take place and that the inability of the cell-free extracts to oxidize ammonia may be due to disruption of energy-coupling mechanisms; this suggestion might also explain the loss of ammonia-oxidizing

ability by "aged" cells. Little is known of the carbon metabolism of *Nitrosomonas* save that it derives its carbon from carbon dioxide and will not incorporate carbon from sucrose (20, 21); moreover, it fails to utilize a wide range of sugars and amino acids (22) and is unaffected by additions of various growth factors to the medium (23, 24). Ruban has, however, made the very interesting observation, confirmation of which will be awaited with impatience, that *Nitrosomonas* is capable of splitting guanine, allantoin, and uric acid and then nitrifying the ammonia released (25); the fate of the carbon residues, whatever they may be, is not clear. The suggestion that *Nitrosomonas* is not an autotroph (26) but derives its cell carbon from dust and detritus in the air has met with opposition (20, 21, 27), yet the fact remains that pure cultures of the organism do seem to die out rapidly (11, 28, 29) suggesting that for some reason many strains of *Nitrosomonas* may benefit from association with other organisms. This tendency has always made metabolic studies of the organism tedious and difficult and therefore the isolation (30) of a vigorous strain capable of persisting in apparently pure culture is to be welcomed, although it seems a pity that effectively only one "heterotrophic" medium was used in the tests for purity. It has been the author's growing conviction for some time that several test media must be used, and all must show the absence of heterotrophic growth before an autotrophic culture can be said to be pure; in this respect a recent isolation of *Nitrobacter* (31) is exemplary.

NITROBACTER

Aleem & Alexander (32) have studied nitrite oxidation in the "red particle" fraction prepared from *Nitrobacter* by sonic disintegration; all the nitrite oxidized could be recovered as nitrate, and oxygen consumption during the oxidation was equal to the theoretical value. There was no evidence of nitrite toxicity even at concentrations of 250 μ M of nitrite per Warburg vessel. Butt (33) also found the nitrite oxidizing activity of the organism was concentrated in an insoluble fraction (prepared by disrupting the cells in a vibrating disintegrator) which he believed to consist mainly of cell walls and adhering cytoplasmic membranes. The cytochrome intimately concerned in nitrite oxidation (34) has been partially purified (35) and some of its physical characteristics determined and compared with those of mammalian cytochrome-*c*. It has an E_o' of +0.25 v. which is much the same as that of mammalian cytochrome-*c*, shows absorption bands at 550 $m\mu$, 521 $m\mu$, and 416 $m\mu$ in the reduced state, but has an isoelectric point in the region of pH 6.5 compared with the pH 10.65 of mammalian cytochrome-*c*. Despite the difference in isoelectric points of the two cytochromes, the cytochrome oxidase of *Nitrobacter* can apparently couple with mammalian cytochrome-*c* during ascorbate oxidation. The part played by cytochromes in nitrite oxidation has been confirmed by Zavarzin whose work also implicates other respiratory pigments (36, 37). His conclusion that catalase is not concerned in nitrite oxidation is in harmony with previous findings obtained by different tech-

niques (16). H. Engel *et al.* report (38) that 2,4-DNP inhibited the growth of *Nitrobacter* without markedly affecting nitrite oxidation; on the other hand, Butt (33) found that 1.5 mM 2,4-DNP reduced the nitrite-oxidation rate of whole cells by 50 per cent. Aleem (39) found that molybdate stimulated the growth of *Nitrobacter*, while Zavarzin (37, 40) reported that iron, together with either molybdate or tungstate, stimulated nitrite oxidation but that if both molybdate and tungstate were used together with the iron they antagonized one another. The effects of the molybdate and tungstate varied with the pH of the medium and, in order to explain his results, Zavarzin suggests that the biologically active form of these ions is a heteropoly ion (presumably phosphomolybdate or phosphotungstate) formed when the ions are added to the culture medium; when both ions are present a mixed, biologically inactive heteropoly ion is formed. He was originally of the opinion (36) that there might be two pathways of nitrite oxidation in *Nitrobacter*, one involving a molybdenoflavoprotein and the other involving the cytochrome system but, in view of the simultaneous requirements for iron and molybdate, he now believes that the molybdenoflavoprotein and cytochrome act sequentially as they do in the terminal respiration systems of other organisms.

A curious effect on nitrite oxidation of ions similar in structure to nitrite has been noted (33, 41). These ions, nitrate, arsenite, and cyanate are to various degrees inhibitory to nitrite oxidation at normal oxygen tensions, cyanate being especially so (34); but as the oxygen tension is lowered, the inhibition lessens and finally, at very low oxygen tensions, gives way to stimulation. It is thought that at normal oxygen tensions the ions act as simple competitive inhibitors but that at low tensions, where nitrite oxidation is in any case slow, they prevent nitrite from accumulating too rapidly at the surface of the oxidizing enzyme systems and thus prevent poisoning of the enzyme systems by excess nitrite.

Little is known about the assimilatory processes in *Nitrobacter*. During nitrite oxidation carbon dioxide is certainly assimilated (42) and inorganic phosphate is esterified (33). However, with the advent of growth techniques capable of producing reasonable yields of the organism (43, 44) we may perhaps soon hope for more detailed analyses of the assimilatory processes.

THIOBACILLI

Oxidation mechanisms.—In their excellent review of the thiobacilli, Vishniac & Santer (45) preface the section on "Oxidation of sulfur compounds" with the remark that "... the intermediate compounds formed, and the relation of oxidative steps to energy storing and energy utilizing reactions are still largely unknown." While this remains true, nevertheless in the two years that have elapsed since the review was published some significant results have appeared that may herald a much better understanding of the oxidative processes.

In 1957, Skarzynski *et al.* (46) by using thiosulfate labelled either in the outer sulfur atom ($-^{35}\text{S}\cdot\text{SO}_3^-$) or the inner one ($-\text{S}\cdot^{35}\text{SO}_3^-$) showed that *Thiobacillus thioparus* metabolizes only the outer sulfur atom, the SO_3^-

moiety of the thiosulfate being left in the medium as sulfate. When cells were grown in a medium containing $-\text{S}^{35}\text{SO}_3^-$ the radioactivity of the medium, freed from cells and precipitated sulfur by centrifugation, dropped sharply as growth proceeded until, after some 10 days growth, almost all the radioactivity was concentrated in the cells and precipitated sulfur. Conversely, when cells were grown in $-\text{S}^{35}\text{SO}_3^-$ the radioactivity of the medium (freed from cells and sulfur) remained unchanged throughout growth. This seemed a clear indication that *T. thioparus* removed only the outer sulfur atom of thiosulfate and left the remainder of the thiosulfate in solution as sulfate, thus showing the same selectivity towards thiosulfate as is shown in *E. coli* (47). Further investigations (48) based on chromatographic analyses of cells that had metabolized "outer labelled" thiosulfate for only 10 min., led to the suggestion that "... the outer sulphur atom of thiosulphate reacts with some organic acceptor in the bacterial cells, forming a compound (not yet identified) detectable during the early stages of incubation with thiosulphate." It was further suggested that the postulated organic acceptor reacts with the $-\text{SH}$ groups of compounds other than thiosulfate since it was found that when *T. thioparus* was grown on unlabelled thiosulfate in the presence of S-labelled cysteine the organisms incorporated the labelled sulfur into the same wide range of sulfur compounds found to be labelled after growth in "outer labelled" thiosulfate. Furthermore, when growth took place in the presence of labelled cysteine labelled sulfur and sulfate eventually appeared in the medium. It is claimed that this sulfur and sulfate are the products of the biological oxidation of cysteine sulfur and cannot have arisen by non-biological interaction of cysteine and thiosulfate since such interaction would have given rise to different compounds. This claim is based on the work of Szczepkowski (49) who showed that cysteine reacted with thiosulfate in neutral solution at room temperature to give cysteine and one molecule of H_2S in which all the sulfur arose from the $-\text{SH}$ of the thiosulfate. The reactions proposed are (where $\text{R}\cdot\text{SH}$ represents cysteine):



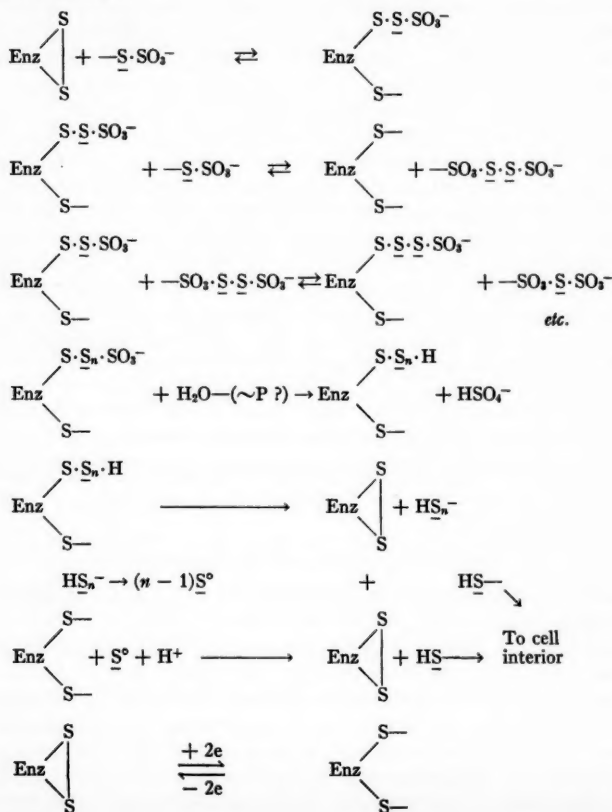
It was further shown, in conformity with what would be expected from the pioneer work of Foss (50), that further reactions could take place at higher temperatures; e.g.,



suggesting the possibility of the synthesis of a whole series of polythiosulfonic acids.

The discovery that *T. thioparus* attacks only the outer sulfur atom of thiosulfate runs counter, it is claimed (46), to the scheme proposed by Vishniac (51) and since slightly extended (45), which calls for the formation of tetrathionate from thiosulfate, the dismutation of the tetrathionate into trithionate and pentathionate, and the breakdown of the pentathionate into tetrathionate and elemental sulfur. If only the outer sulfur of thiosulfate is metabolized and the $-\text{SO}_3^-$ moiety left in solution as sulfate, no tetrathio-

nate will be formed and indeed, more recent work of the Polish group (52) shows that their strain of *T. thioparus* does not normally give rise to any chromatographic spot for tetrathionate during growth. However, when tetrathionate was allowed to diffuse slowly from a dialysis sac into a culture growing on thiosulfate, spots corresponding to tetrathionate, trithionate, and pentathionate were evident after 15 hr. Trudinger, however, has pointed out (53) that the Polish workers were using long periods of growth for their investigations and showed that with *Thiobacillus* X (an organism similar to *T. thioparus*), tetrathionate appeared in high concentrations immediately on the addition of thiosulfate, fell in concentration over the next 60 sec. to be replaced by trithionate and other unidentified compounds in conformity with Vishniac's scheme. It seems possible to harmonize all of these findings if we suppose that on the cell surface there is an enzyme system with a mechanism similar to that proposed for rhodanese by Sörbo [See (54)]. The types of reaction envisaged are:



This formulation, although hypothetical, seems reasonable. It involves the formation of polythiosulfonic acid chains (49) and the eventual splitting off of the terminal —SO_3^- as sulfate, possibly via the intermediate formation of a mixed anhydride of the type $(\text{—S—O—PO}_4=)$ envisaged by Vishniac & Santer (45). The residual enzyme-polysulfide complex, which is similar to that found in rat liver (55), then breaks down to elemental sulfur and sulfide (both derived entirely from the 'outer sulfur' of thiosulfate) and the sulfide alone is transported into the cell. The sulfur is eventually reduced to sulfide by the same enzyme system, the electrons necessary for the reduction being supplied by the intracellular oxidation of the sulfide. This scheme is similar to the one already suggested (45) with the slight modification that polythionate formation is thought of here as an enzyme-controlled process on the cell surface, although chemical dismutations between polythionates in the medium (45) are, of course, not ruled out. It has the negative point in its favor that it could not account for the oxidation of dithionite which is only slowly (45) oxidized by thiobacilli if it is oxidized at all (56). The behavior of different thiobacilli with regard to the formation of sulfur and polythionates can be explained by assuming that they split the polythiosulfonic acid chain at different chain lengths. If the chain is split when it is simply —S·S·SO_3^- , no polythionate or sulfur appear; if the S^0 is reduced very rapidly at the enzyme surface, no sulfur appears. Various permutations of chain length and speed of sulfur reduction could probably account for all the variations in behavior of different thiobacilli towards different sulfur compounds noted by Parker & Prisk (57), the Polish school, Trudinger, and Vishniac & Santer. The suggestion that sulfur is reduced to sulfide at the cell surface is in agreement with the discovery by Suzuki & Werkman (58) that cell-free extracts of *T. thiooxidans* oxidize sulfur only when reduced glutathione is present and they suggest that "Elemental sulfur might enter the cell by forming —SS— bonds with the sulphydryl groups of protein or glutathione located near the surface of the cell. It is also possible that sulfur is reduced to hydrogen sulfide by glutathione outside the cell and enters the cell in the form of the soluble sulfide ion." Therefore, if the suggested scheme is approximately correct, all thiobacilli oxidizing sulfide, sulfur, thiosulfate, or polythionate are really oxidizing, within the cell, the same sulfur compound; namely, sulfide, and interest therefore centers on how this particular oxidation takes place. Unfortunately, mechanisms of biological sulfide oxidation remain somewhat obscure although there has been much recent activity in this field (54). It seems that sulfite may be an intermediate (45) and it is known that some thiobacilli will oxidize sulfite directly (57); sulfite has also been suggested as an intermediate in the formation of thiosulfate from sulfide in rat liver (59, 60) in which a protein-bound intermediate, possibly a thiosulfonate, was detected.

Energy sources used by thiobacilli.—*Thiobacillus novellus*, originally isolated by Starkey and subsequently lost, has been reisolated and studied by Santer *et al.* (61) who have shown that each cell of the organism can grow on nutrient broth, on a glutamate-citrate-salts medium, or autotrophically on thiosulfate. *Thiobacillus ferrooxidans*, whose existence has been somewhat

doubted since the isolation of *Ferrobacillus ferrooxidans* (62), apparently does exist and oxidizes Fe^{2+} to Fe^{3+} while assimilating CO_2 with the surprisingly high free-energy efficiency of 30 per cent over the first two days of growth (63). This value fell as growth proceeded and cultures two or three weeks old showed free-energy efficiencies of only 4.4 to 5.3 per cent, thus approximating to the original figure of 3.2 per cent found by Temple & Colmer (64) for a 17-day-old culture. The ability to oxidize thiocyanate was thought to be confined, among the thiobacilli, to the *T. thiocyanoxidans* of Happold & Key (65) until de Kruffy *et al.* (66, 67) showed that *T. denitrificans*, *T. thioparus*, and *T. thiocyanoxidans* all oxidize thiocyanate aerobically to sulfur and sulfate. The last two organisms also grew anaerobically on thiosulfate or thiocyanate with nitrate as electron acceptor; the nitrate was reduced to nitrite during growth and not to the N_2 gas produced by *T. denitrificans* under similar cultural conditions. These results, de Kruffy and her colleagues suggest, raise the question whether *T. thiocyanoxidans* should be regarded as a separate species since it appears identical with *T. thioparus*. Happold *et al.* tend to agree as far as the present strain of *T. thiocyanoxidans* is concerned (68) but stress the point that there have been metabolic differences between different strains isolated from time to time. It appears that polythionate formation is more marked in cultures of *T. thioparus* than in cultures of *T. thiocyanoxidans* (69), a point adumbrated by the work of Pratt (70). A second point of interest is that cells grown on thiosulfate will not oxidize formate or succinate to any appreciable extent; whereas, cells grown on thiocyanate will do so, formate oxidation being especially marked. Cultures with a high endogenous respiration will also oxidize other substrates such as pyruvate (68).

Cytochromes and the generation of bond energy in thiobacilli.—Trudinger (71) has demonstrated coupling between thiosulfate oxidation and cytochrome reduction in *Thiobacillus* X. When thiosulfate was added to whole cells or crude extracts, spectroscopic examination of the cells or extracts showed the presence of reduced cytochromes. Four cytochrome fractions were prepared by ammonium sulfate fractionation followed by adsorption from phosphate buffer onto an Amberlite IRC 50 column. All four yielded hemochromogens of the *c* type, but each differed in some particular from the cytochrome-*c* of *T. denitrificans* (72) and the single cytochrome-*s* of *T. thioparus* (73), which is claimed to be autooxidizable and thus resembles the 553.5 cytochrome of *Thiobacillus* X although differing from it in oxidation-reduction potential. The 553.5 cytochrome of *Thiobacillus* X was found to react very rapidly with an enzyme system isolated simultaneously (and apparently containing no cytochrome) that oxidized thiosulfate to tetrathionate with the simultaneous reduction of ferricyanide or the cytochrome. The other three cytochromes reacted only slowly with the thiosulfate-oxidizing enzyme unless a trace of the 553.5 cytochrome were present. Szczepkowski & Skarzynski (74), while able to demonstrate cytochrome, cytochrome oxidase, catalase, and (rather less well) peroxidase activity in *T. thioparus* could find no indica-

tion of any of the first two and the last of these systems in *T. thiooxidans*, although catalase activity was present. While the conclusion drawn by these workers, that *T. thiooxidans* is "... the unique obligate aerobic micro-organism which possesses no cytochrome system and consequently cannot carry out oxidation mechanism of the type common for all other aerobic species" may be correct; if it is correct then it argues a biochemical situation so extraordinary that it merits much more investigation.

Milhaud *et al.* (75) have shown that *T. denitrificans* incorporates inorganic phosphate into ATP only when thiosulfate oxidation is proceeding and, indeed, it must be generally accepted that oxidation of sulfur compounds by thiobacilli is accompanied by the generation of $\sim P$ and reducing power, although the extent to which the reducing power and $\sim P$ can be stored has given rise to some controversy in the past. Briefly, the position in 1955 was (3) that Vogler & Umbreit (76) had found the storage to be considerable; indeed, so considerable that when their results were examined quantitatively it was found that they were explicable only if it was assumed that all the phosphate in the cell was in the form of ATP.

These quantitative difficulties seem now to have arisen from a certain misapplication, unsuspected at the time, of the dry weight versus optical density curves used to assess the dry weight of bacteria in the Warburg vessels. In fact, Vogler & Umbreit were probably using a weight of bacterial cells considerably greater than that reported (77). The report of LePage & Umbreit (78) that the ATP of *T. thiooxidans* was 3', not 5' ATP, could not be confirmed by Barker & Kornberg who found, by means of column chromatography and subsequent specific enzymic analysis, that at least 80 per cent of the ATP of *T. thiooxidans* was the normal 5' ATP (79). However, the subsequent discovery of phosphoadenosine phosphosulfate as the active form of sulfate in many cells makes it just possible that this compound plays a part in the sulfur economy of *T. thiooxidans* and that LePage & Umbreit happened to examine their organisms when the concentration of this compound was reasonably high.

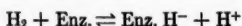
FERROBACILLUS FERROOXIDANS

This organism has recently been investigated by Silverman & Lundgren (80, 81). Rigorous attention to keeping the cultures in a state of physiological youth combined with forced aeration of the cultures resulted in good growth while a simple method of sedimentation separated the cells fairly easily from precipitated ferric salts. Manometric studies on whole cells showed that oxygen uptake during oxidation of ferrous salts was 92 per cent of the theoretical value. Optimal conditions for the oxidation were reported to be at pH 3.0 to 3.6 and 37°C. although no growth took place at this temperature. There was no evidence of iron toxicity even at iron concentrations of 500 μM per Warburg flask. Resting cells were unable to oxidize ammonia, thiosulfate, or salts of manganese, cobalt, or nickel; they were able, however,

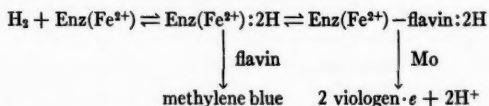
to oxidize elemental sulfur slowly. Estimates of free-energy efficiency made during the oxidation of 50 μ M of iron gave a value of 20.5 ± 4.3 per cent which is close to that found for *T. ferrooxidans* in young vigorous cultures (63).

HYDROGENOMONAS

There has been considerable interest in the biochemistry of *Hydrogenomonas* species, if indeed such a species epithet is really permissible (82). The action of hydrogenase has been investigated in work not confined to the hydrogenase from autotrophic bacteria but which must obviously be taken into account when autotrophic oxidation of hydrogen is being considered. Krasna & Rittenberg (83), working with the hydrogenase of *Proteus vulgaris*, studied the mechanism of its action in catalyzing the *ortho-para* conversion of hydrogen when the *para*-hydrogen content of the gas phase was augmented by *para*-hydrogen prepared by adsorbing ordinary hydrogen onto charcoal at low temperatures. Experiments were also carried out to measure the catalytic effect of hydrogenase on the exchange of deuterium, present in the gas phase as D_2 , with the hydrogen of water, and on the exchange of *para*-hydrogen in the gas phase with the deuterium of D_2O . On the basis of their results they suggested that the action of hydrogenase could be formulated as:



Peck *et al.* (84), on the basis of experiments carried out with the then new technique of hydrogenase-catalyzed release of hydrogen from viologen dyes (85), thought the reaction was better described as:



Krasna & Rittenberg (86) subsequently suggested that while the activation of hydrogen, as measured by exchange reactions, was probably a fairly simple process, the utilization of the activated hydrogen for actual reductions might require cofactors or additional enzyme systems. Packer & Vishniac (87) partially purified the hydrogenase from *Hydrogenomonas ruhlandii* and found that utilization of hydrogen was specifically linked to diphosphopyridine nucleotide reduction; TPN would not replace DPN and the preparation did not reduce oxygen. Atkinson (88) found that growth of *Hydrogenomonas facilis* was inhibited by low levels of nitrate and concluded, from the results of experiments on the reduction of methylene blue, that the active inhibitor was nitric oxide formed during the reduction of the nitrate. Nitrate, nitrite, and hydroxylamine had no effect on methylene blue reduction but hydroxylamine inhibited the reduction of oxygen. Atkinson & McFadden (89) succeeded in preparing cell-free extracts of *H. facilis* in which the hydrogenase activity was particle-bound and which retained the ability to catalyze the

reduction by hydrogen of methylene blue, ferricyanide, and oxygen. These workers, after careful consideration of the effects of various enzyme inhibitors on their preparations, remained uncertain whether or not iron played an essential part in the enzyme system. Packer (90) identified, by their absorption spectra, a number of respiratory pigments in cells of *H. facilis*; these included flavins, cytochromes, and two pigments binding carbon monoxide. Addition of lactate to, or the introduction of hydrogen into, cell suspensions caused a change in the level of reduced cytochrome-*c* and this change was related to the respiration rate. Azide and cyanide accelerated the rate of cytochrome-*c* reduction while Antimycin-A and amobarbital sodium (Amytal) decreased it. These inhibitors acted similarly whether hydrogen or lactate was the hydrogen donor, suggesting there was no qualitative difference between the electron transport paths for the two donors. However, the actual rate of respiration with the two donors and the concentration of the two CO-binding pigments depended on the previous cultural history of the cells. Atkinson (91) found that freshly harvested autotrophically grown cells of *H. facilis* would not oxidize glucose and that various metabolic acids were oxidized only after a lag period but thereafter at increasing rate. This seemed to be a case of enzyme adaptation since an exogenous supply of nitrogen enhanced the ability of the cells to deal with organic substrates while 2,4-DNP diminished it; whether or not they had become adapted to organic substrates the cells could always carry out a rapid oxidation of hydrogen the rate of which remained constant. Lindsay & Syrett (92), however, found that heterotrophically grown *H. facilis* lacked hydrogenase activity but that the activity could be induced by exposing the cells to an atmosphere of 95 per cent hydrogen and 5 per cent air. Induction of the activity was inhibited by chloramphenicol and 2,4-DPN; it was also inhibited by lactate and acetate unless a nitrogen source such as ammonium sulfate was present.

ASSIMILATION OF CARBON DIOXIDE BY CHEMOLITHOTROPHS

In 1955, Santer & Vishniac (93) found that ribulose diphosphate stimulated the incorporation of the carbon of $^{14}\text{CO}_2$ into crude extracts of *Thiobacillus thioparus*; all the carbon so incorporated was found in the carboxyl group of phosphoglyceric acid. Simultaneously, Trudinger reported that the incorporation of carbon from $^{14}\text{CO}_2$ into extracts of *T. denitrificans* was stimulated by ribose-5-phosphate and ATP (94); ammonium sulfate fractionation of the extracts yielded a preparation in which carbon incorporation was dependent upon the simultaneous presence of ribose-5-phosphate and ATP, and analyses showed that 93 per cent of the incorporated carbon was present in phosphoglyceric acid. Later work (95) proved that ribulose diphosphate could replace ribose-5-phosphate plus ATP, that the extracts contained most of the enzymes of the Calvin cycle, and that they converted hexose diphosphate into a mixture of pentose, heptose, and tetrose. Trudinger therefore concluded that *T. denitrificans* is capable of synthesizing hexose phosphates

from carbon dioxide by a cyclic mechanism similar to that found in green plants. Subsequent work by the French school (75; 96 to 99) with *T. denitrificans* has confirmed these findings and has demonstrated (97) that carbon incorporation in whole cells is dependent on thiosulfate oxidation; washed cells suspended in bicarbonate labelled with ^{14}C did not incorporate the label in the absence of thiosulfate. The later work of this school (99) suggested that a second, minor carbon fixation process was operating in the organisms and fixing carbon by the carboxylation of phosphoenolpyruvate to yield oxalacetate. This second mode of fixation, which is known to occur in some plants (100) has also been found to operate in *T. thiooxidans* (101, 102) and it was tentatively suggested that since the Michaelis constant of the phosphoenolpyruvate carboxylating enzyme with respect to bicarbonate is about one-tenth of that of the ribulose diphosphate carboxylating enzyme, assimilation of carbon via the phosphoenolpyruvate carboxylation might be favored at the low pH's normal in *T. thiooxidans* cultures and in which bicarbonate concentrations are consequently low. However, later work (103, 104) showed that in a 2-sec. assimilation period 80 per cent of the carbon assimilated was incorporated into phosphoglyceric acid and only 8 per cent into the aspartic acid derived from oxalacetic acid. The assimilation of carbon dioxide into the expected phosphoglyceric acid has been shown for *H. facilis* by Bergmann *et al.* (105) who also noted a certain amount of fixation into C_4 dicarboxylic acids, presumably by some such mechanism as the carboxylation of phosphoenolpyruvate. McFadden (106) found that phosphoglyceric acid, fructose-6-phosphate, and ribulose diphosphate were the products of 25-sec. and 45-sec. assimilations of labelled carbon dioxide by *H. facilis* under autotrophic conditions; under heterotrophic conditions the products were the same but were less extensively labelled. He also noted that 2,4-DNP (0.2 mM), which did not prevent the oxidation of hydrogen from taking place, nevertheless blocked the formation of pentose, hexose, and heptose phosphates in a 45-sec. autotrophic assimilation of labelled carbon dioxide, although phosphoglyceric acid itself was formed. McFadden notes that ATP is necessary for the reductive conversion of phosphoglyceric acid to triose phosphate and suggests that in the experiments just quoted, the 2,4-DNP was exerting its usual uncoupling effect and so limiting the supply of ATP. Faust (107), studying the formation of labelled amino acids from $^{14}\text{CO}_2$ by *H. facilis* under autotrophic conditions found that, after 6 sec. exposure, glutamic acid, glycine, serine, and aspartic acid were all labelled and that there was a trace of the label in the leucine-isoleucine fraction. Several possibilities were considered but the early appearance of, and continuing high level of, labelling in the glutamic acid were thought to indicate that there was carboxylation of a C_4 compound to yield α -ketoglutarate while the early appearance of labelled glycine and serine, coupled with the finding that the glycine was labelled predominantly in the carboxyl group, pointed to the operation of a normal Calvin cycle. It was concluded that carbon dioxide fixation in *H. facilis* took place by more than one pathway.

Orgel (108), also working with *H. facilis*, found that the phosphate ester and steam volatile fractions of cells that had carried out a 6-sec. fixation of $^{14}\text{CO}_2$ contained 50 per cent of the labelled carbon that had been incorporated and that in the steam volatile fraction the formic acid was more strongly labelled than the acetic. It does indeed seem possible that "minor" pathways of carbon assimilation may be more marked in *Hydrogenomonas* species than in other autotrophic organisms, although it appears probable that even here the main pathway of fixation is similar to that followed in green plants. This conclusion was reached by McFadden & Atkinson (109) who made a detailed study of the factors affecting fixation of carbon dioxide by whole cells of *H. facilis*. They took considerable precautions to allow for the effects of the high endogenous respiration of the cells and compared the effects of different poisons on hydrogen oxidation and carbon dioxide fixation as measured by the increase in radioactivity of the cells when oxidizing hydrogen in the presence of $^{14}\text{CO}_2$ in a medium of phosphate buffer plus ammonium chloride, omission of which from the medium reduced fixation by 30 to 60 per cent. Small volumes of a dilute suspension of cells were used to ensure adequate aeration. Fixation was inhibited by *p*-chloromercuribenzoate, versene, 2,2'-dipyridyl, and thioglycollic acid at concentrations having no effect on hydrogen oxidation. Since the effects of these poisons on the assimilation of carbon dioxide in *H. facilis* proved similar to their effects on the same process in chloroplast preparations, it was concluded that the mechanisms of fixation in chloroplasts and *H. facilis* were probably also similar.

In completing this section, I can only regret my inability to understand the work which purports to show that hydrogen bacteria grow best when supplied with both carbon dioxide and bicarbonate (110). This appears to be based on the belief that in a closed system containing a buffered solution and a gas phase it is possible to set the equilibrium value of the ratio "(partial pressure of CO_2 in the gas phase):(concentration of dissolved CO_2):(concentration of HCO_3^-)" at any figure without reference to the pH of the solution and, in particular, on the belief that the first term can be reduced to zero without affecting the magnitude of the last.

OTHER POSSIBLE CHEMOLITHOTROPHS

Desulfovibrio species were excellently reviewed by Postgate (111) last year. Since that review, two papers have appeared that are germane to this. Sadana & Morey (112) described the purification of a hydrogenase from *Desulfovibrio desulfuricans*, with a specific activity of $11.4 \times 10^6 \mu\text{l. H}_2$ absorbed/hr./mg. enzyme-N in the methylene blue assay, that was completely inactive in that assay in the absence of iron. Mechals (113) showed that growth of the same organism under strictly autotrophic conditions was scanty and that carbon incorporation from $^{14}\text{CO}_2$ did not exceed 25 per cent of the total carbon incorporated, the rest being provided presumably from traces of organic materials in the "inorganic" medium. These observations have been confirmed by Postgate (114) and support his suggestion that

Desulfovibrio species are probably not fully autotrophic (111). Whether bacteria that assimilate carbon dioxide by utilizing energy from radioactive decay should be classed as chemolithotrophic or photosynthetic is an interesting question. It is also a purely academic one since, apparently, they do not exist (115).

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INDUSTRIAL FERMENTATIONS¹

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Since the last general review of Industrial Fermentations appeared in the *Annual Review of Microbiology* in 1953 (1), the literature on the subject has become so voluminous that in a review of this kind it is impossible to discuss other than a few of the more significant contributions to the field. Since the 1953 report over 2000 papers have appeared which dealt wholly or in part with the subject of industrial fermentations. The selection of papers for this review has been simplified by the publication of a number of recent books and reviews. Included in the list are the two volumes of *Industrial Fermentations* (2, 3), *Industrial Microbiology* (4), *Progress in Industrial Microbiology* (5), and *Advances in Applied Microbiology* (6). The comprehensive reviews on fermentations that appear each September in *Industrial and Engineering Chemistry* have been especially useful (7 to 12).

In this review particular attention will be placed on recent publications which contributed to improvement in the production of a specific fermentation product or which contributed basic knowledge to applied microbiology in general.

ANTIBIOTICS

An interesting discussion of the impact of antibiotics on agriculture, medicine, science, and society recently was published by Burkholder (13). The hope was expressed that we may be standing on the threshold of ultimate prevention or cure of all infectious diseases.

Penicillin.—In the past six years a great number of papers have dealt with the various aspects of the production of penicillin. These studies have covered four main areas: (a) the development of high-yielding strains; (b) the effect of environmental factors on penicillin formation; (c) the metabolism of *Penicillium*; and (d) the biosynthetic pathway in the formation of the penicillin molecule. Detailed discussions of these four areas may be found in recent reviews by Arnstein & Grant (14), Jackson (15), Hockenhull (16), Demain (17), and Chain (18).

The most important factor responsible for the tremendous increase in the efficiency of the penicillin fermentation in the last fifteen years has been the selection of high-yielding strains of the *Penicillium notatum-chrysogenum* group. Backus & Stauffer (19) have described the development from *P. chrysogenum* X-1612 of mutant strains capable of producing 3000 units of penicillin per ml. A tenfold increase in titers was obtained over a ten-year period and, in addition, the newer strains produced mostly penicillin G and they did not produce any of the troublesome yellow pigment so characteristic

¹ The survey of the literature pertaining to this review was concluded in December, 1959.

of Q 176 and earlier strains. A progressive decline in the vegetative and reproductive vigor of the strains accompanied their increased ability to synthesize penicillin.

The "Wisconsin family" of pigmentless strains was secured through spontaneous changes or through treatment of spores with ultraviolet radiation and with methyl-*bis* (β -chloroethyl) amine. The search for new and improved strains is a major activity of pharmaceutical companies and until recent years this search, of necessity, was patterned after the time and labor-consuming "hit or miss" method used to obtain the "Wisconsin family" strains. Pontecorvo (20, 21) recently described a "parasexual" breeding technique in which strains of microorganisms with desired genetical factors are combined. Investigations on the production of new penicillin-producing strains have been discussed by Kleiner & Ioffo (22) and Caglioti & Sermoni (23), and reviews on the genetics of fungi have been prepared by Pontecorvo (20), Cavalli-Sforza (24) and Wheeler (25). A monograph on the genetics of *Streptomyces* and other antibiotic-producing organisms recently appeared (26).

Since 1953 there have been few papers on the effects of fermentation variables on the production of penicillin. Bhuyan & Johnson (27) found that by a proper balance of nutrients it was possible to obtain high penicillin titers, even in the absence of corn steep liquor, with distillers solubles, cottonseed meal, and soybean meal. Anderson, Tornqvist & Peterson (28) obtained penicillin titer increases over controls of almost 150 per cent when they made intermittent additions of lard oil to 50-gallon pilot tank runs. The added oil served as a nutrient, as an antifoam agent, as a buffer, and it extended the productive phase of the fermentation. In an extension of these studies it was found that the effect of oil in either a standard or a rich medium depended upon the amount of CaCO_3 present. The oil depressed the pH and penicillin titers if less than 0.4 per cent CaCO_3 was present (29). Macmorine (30) reported that unsaturated oils stimulated production of penicillin F, whereas saturated oils stimulated production of dihydro-F and K. Lard oil favored production of penicillin X but penicillin G titers were not markedly affected by the presence or absence of either lard oil or soya bean oil. Pan and his co-workers at Squibb (31) observed that with the proper oil addition schedule of an optimum ratio of mineral oil to corn oil it was possible to obtain penicillin yields almost twice those obtained from lactose controls. Hockenhull at the Glaxo Laboratories recently has discussed the possible roles of the lipid nutrient on penicillin biosynthesis (16). He speculated that the fatty acid served to supply excess acetyl-coenzyme A to the tricarboxylic acid cycle. The excess thus exerted a back pressure on the thioctic acid-catalyzed oxidation of pyruvate or acetaldehyde. These metabolites may then form the 5-carbon skeleton of penicillamine. Other possible roles were suggested.

In a study of the effects of the fermentation temperature on penicillin production, Owen & Johnson (32) showed that there were two optima. One,

about 30°C., was best for the mycelium-producing phase; the other, about 20°C., was best for the antibiotic-producing phase. This observation may be encountered in other fermentations where the growth phase and product-forming phase are physiologically distinguishable.

Two comprehensive discussions on the pilot-scale production of penicillin have appeared. Chain edited a book on the pilot plant techniques of submerged fermentation in which he discussed various phases of work conducted at the Istituto Superiore di Sanita (18). In 1958 Steel edited a book, *Biochemical Engineering*, which covers nearly all unit processes in industrial fermentation (33). A section of the book was devoted to the development of the penicillin fermentation (15).

There have been very few papers on the production of penicillin by the continuous fermentation process despite the fact that the production of penicillin has been highly competitive. Stimulus for further study in this area may come from recent work reported by Bartlett & Gerhardt (34) and Pirt & Callow (35).

Much work has been carried out in the last six years on the mechanism of penicillin biosynthesis. Excellent reviews by Hockenhull (16) and by Demain (17) appeared on the subject in 1959. Details of the biosynthetic work will not be discussed here. In brief summary, the sulfur atom, the side-chain nitrogen atom, and the carbon and hydrogen atoms of the β -lactone come from L-cysteine. The penicillamine portion of the molecule is probably synthesized from the entire L-valine molecule although some controversy exists regarding the incorporation of the amino group of L-valine. Very little is known about the mechanism of the inversion to the D structure of penicillin or about the mechanism of condensation of valine and cysteine. The rate-limiting step in the biosynthesis of penicillin is the addition of the side-chain and to obtain high titers a suitable precursor is added to the fermentation medium.

One of the recent clinically effective penicillins, penicillin V, was obtained from a study of additions of various precursors to *P. notatum* fermentations (36). Not only is the V more acid-stable than penicillin G but, in addition, higher blood levels are obtained with the same dosage. A recent announcement from the Beecham Research Laboratories has created a renewed interest in new clinically active penicillin analogues (37). These workers isolated 6-aminopenicillanic acid from a *P. chrysogenum* W-51-20 fermentation carried out in the absence of added precursor. The isolation of this acid suggests that the last step in the biosynthesis of penicillin is the acylation of the nucleus. Kato appears to have been the first person to describe the accumulation of 6-aminopenicillanic acid in penicillin fermentation broths (38). However, he did not isolate or identify the compound.

Tetracycline.—The tetracyclines, a group of structurally related antibiotics widely used in the treatment of infectious diseases, differ in their antimicrobial activity and in their therapeutic action (39, 40). The anti-



microbial activity of the tetracyclines has been ascribed by Weinberg to their ability to chelate essential metabolic cations of the susceptible microorganism (41). A comprehensive review of the tetracycline fermentations was prepared by Di Marco & Pennella (42).

The United States Tariff Commission, Washington, D. C., reported that in 1958 243,000 lbs. of tetracycline were sold in the United States at a value of 105 million dollars. Thus, tetracycline had the greatest sales value of any American fermentation product.

A number of cultures have been reported that produce tetracycline. Among them are three unnamed Pfizer strains, *Streptomyces aureofaciens* from Lederle, *Streptomyces viridifaciens* from Bristol, and *Streptomyces feofaciens* from Lepetit S.P.A. Di Marco & Pennella have described and compared the cultural characteristics of these strains (42).

One of the problems associated with the production of tetracycline is the co-production of chlortetracycline. To obtain tetracycline the following specific fermentation conditions have been used: (a) dechlorination of the natural products media by precipitation (43) or by treatment with ion exchange resins (44, 45); (b) inhibit utilization of the chloride of the natural products media by either bromide, iodide, thiocyanate, or a number of sulphydryl-containing compounds (46, 47, 48); (c) use of chloride-free, chemically defined media (45) or dechlorinated corn steep liquor plus oil-free cottonseed endosperm flour (44). A patent which describes the chemical production of tetracycline from chlortetracycline has been issued to Conover (49).

Chlortetracycline.—There have been few papers on the selection of high-yielding mutants for production of chlortetracycline. Katagiri (50) obtained a high-yielding strain by ultraviolet irradiation of a strain pretreated with chlortetracycline-containing agar. It was observed that strains that were highly pigmented during growth were also the best chlortetracycline producers.

The complex chlortetracycline molecule has been shown by the ^{14}C -incorporation studies of Miller *et al.* (51) to be synthesized from a simple medium composed of glycerol, ammonium ions, and salts. When uniformly-labelled starch was added to the medium it was noted that almost 90 per cent of the chlortetracycline carbon came from the starch. The effect of phosphate on the biosynthesis of chlortetracycline has received much attention. Di Marco (52) reported that the addition of 0.03 per cent K_2HPO_4 to a medium increased sucrose consumption rate, caused a transitory increase in pyruvic acid in the medium, decreased the dry weight of cells, changed the chemical composition of the mycelium, and markedly depressed chlortetracycline production. It was thought that the addition of phosphate to the medium stimulated glycolysis and thus removed from the hexose monophosphate pathway carbohydrate required for synthesis of large amounts of chlortetracycline. Prokoféva-Belgovskaya & Popova (53) reported that batches of corn steep liquor which produced little antibiotic contained high levels of

inorganic phosphate, and Doskočil *et al.* found that chlortetracycline production did not occur until the inorganic phosphate of the medium had been consumed (54). Adverse effects of phosphate were found to be less in tanks than in shaken flasks because of the higher oxygen transfer rate in tanks.

Chlortetracycline titers of 5500 $\mu\text{g.}$ per ml. have been claimed in a patent issued to Goodman (55). The high yields were obtained from a medium which contained as energy sources 60 gm. per l. starch and 30 ml. per l. lard oil. Tetracycline yields of 5600 $\mu\text{g.}$ per ml. were obtained from a chloride-free medium of similar composition.

In pilot plant tanks Mátelová and co-workers (56) observed that intermittent interruption of agitation depressed chlortetracycline yields to 20 per cent of the uninterrupted control yields. The timing concerned with the agitation interruption was critical. Herold *et al.* described conditions in which it was possible to run fermentations with *S. aureofaciens* without maintaining aseptic conditions (57).

A very interesting patent application recently has been opened for public inspection on the production of a "Cosynthetic Factor-1 (CF-1) (58). The CF-1 which is produced by a number of *Streptomyces* species has the property of stimulating the production of high concentrations of chlortetracycline or tetracycline when it is added to a fermentation by strains of *S. aureofaciens* which ordinarily produce minimal amounts of either antibiotic. The CF-1 does not appear to be a precursor.

Oxytetracycline.—This broad-spectrum antibiotic is produced by *Streptomyces rimosus*, *S. platensis*, *S. vendargensis*, and *S. gilvus* (42). A unique feature of the oxytetracycline-producing strains is their inability to utilize sucrose as a carbon source. In culture development Borenstajn & Wolf used a combination of ultraviolet radiation and methyl-bis (β -chloroethyl) amine treatment of an *S. rimosus* strain to obtain cultures that produced up to 2000 mg. oxytetracycline per l. (59). They found that no relationship existed between morphological or cultural characteristics and antibiotic production but it was observed that high-yielding isolates, in contrast to low-yielding ones, readily utilized soybean oil or peanut oil. Alikhanian *et al.* have reported that a number of *S. rimosus* strains that they obtained by either mutation or hybridization required increased amounts of inorganic phosphorus in the medium for maximum production of oxytetracycline (60).

A very interesting paper on the biochemistry of the *S. rimosus* fermentation in relation to the production of oxytetracycline was prepared by Doskočil *et al.* (61). They found about 1 mg. per l. of sodium pyruvate in the medium 12 to 15 hr. after inoculation. The production of the pyruvate occurred during the period of logarithmic growth. Between 15 and 30 hr. the pyruvate was consumed, fragmentation of the non-growing mycelium occurred, and very little oxytetracycline was produced. From the fragments of the Gram-positive primary mycelium, Gram-negative secondary mycelium appeared. Oxytetracycline was then produced at a rate of about 12 mg. per l. per hr. for the next 80 hr. Russian workers recently described a chemically defined medium for an

S. rimosus culture from which they obtained up to 1900 mg. per l. oxytetracycline in 4 to 5 days (62). The medium was composed of starch, glucose, $(\text{NH}_4)_2\text{SO}_4$, NH_3 , succinic acid, and salts.

Demethylchlortetracycline.—The most recent addition to the tetracycline group of clinically useful antibiotics is 7-chlor-6-demethyltetracycline (39). This antibiotic is produced in a chloride-containing medium by a mutant strain of the *S. aureofaciens* parent strain A-327 isolated by Duggar. The antibiotic produces much higher and longer sustained levels of antibacterial activity in blood after oral administration than either chlortetracycline or oxytetracycline (63).

Streptomycin.—The streptomycin fermentation appears to have received little attention in recent years. Woodruff & McDaniel (64) prepared a comprehensive survey of the streptomycin literature in 1954.

The addition of a number of 5,5-dialkyl-barbituric acids and related compounds to a chemically defined medium has greatly increased streptomycin production according to Ferguson *et al.* (65). In a patent issued to Carvajal average streptomycin yields of 3700 μg . per ml. were claimed when a strain of *Streptomyces griseus* was inoculated to a chemically defined medium composed of dextrose, dextrin, $(\text{NH}_4)_2\text{SO}_4$, and NaNO_3 (66).

A recent paper by Kazanskaia (67) described the effect of soy oil and its components on the production of streptomycin by *Actinomyces streptomycini*. Dramatic drops in antibiotic yield were obtained when defatted soy meal was used to replace the regular non-defatted soy meal. The addition of palmitic acid to the defatted soy meal increased the titers to 94 per cent of those of the controls.

Compared to penicillin there appears to be relatively little known of the mechanism of the biosynthesis of streptomycin. Hunter & Hockenhull obtained a 5 per cent incorporation of D- ^{14}C glucose into streptomycin by adding the labelled material after maximum cell growth had been obtained at about 60 hr. (68). Glucose appeared to supply *S. griseus* with all the carbon atoms necessary for the elaboration of streptomycin.

In 1957 Tatsuoka *et al.* reported on the production of dihydrostreptomycin by a new species, *Streptomyces humidis* (69). Dihydrostreptomycin is produced commercially by the reduction of streptomycin.

Other antibiotics.—At least nine other antibiotics have attained varying degrees of clinical importance in recent years. They are bacitracin, erythromycin, kanamycin, neomycin, novobiocin, nystatin, polymyxin B, oleandomycin, and tyrothricin. Chloramphenicol has become a very successful broad spectrum antibiotic; however, it is manufactured by chemical synthesis. In 1959, griseofulvin received considerable attention because of promising antifungal activity in humans. Cycloheximide has received increased attention because of its activity against a variety of plant fungal diseases. Specific information on the production or the activity of these products may be obtained from references listed in the *Industrial and Engineering Chemistry*

series (7 to 12) or from the *Antibiotics Annual* series (70). A survey of the non-therapeutic uses of antibiotics has been prepared by Goldberg (71). Increased use of antibiotics for food preservation and for control of plant diseases can be expected.

VITAMINS

Cobamide (vitamin B₁₂). In 1957 the United States Tariff Commission reported that 537 lbs. of cobamide were sold at a value of almost 22 million dollars. A comprehensive survey of the literature on the microbial synthesis of natural and "unnatural" cobamides has been prepared by Perlman (72). In addition, a timely discussion on the nomenclature and on analytical methods for the determination of the cobamides was presented.

Many genera of bacteria and various streptomycete species are known to produce significant quantities of various cobamides (72, 73). Baker & Ross (74) claimed that they were able to obtain up to 6 mg. of cobamide per l. with certain thermophilic *Bacillus* species, and Rickes & Wood (75) reported that significant levels of cobamide were produced by five different *Streptomyces* species. There have been few reports on mutation studies designed to obtain high-yielding cobamide strains. Pagano & Greenspan (76) described a procedure used to obtain a mutant from an unidentified streptomycete which produced 5.7 mg. cobamide per l. of a soybean meal, glucose, and salts medium. This yield was almost three times that obtained from the parent culture.

Kurz & Nielsen studied the effect on the production of cobamides of additions of various amino acids to a glucose and salts basal medium (77). No direct relationship between the amino acid composition of the medium and cobamide production was observed. There also was no relationship between cobamide production and growth. Perlman and co-workers (78) observed that changes in the type or quantity of energy source in a natural products medium had little effect on cobamide production. However, increases in the natural nitrogen content of the medium increased cobamide production significantly. Similar yields of cobamide were obtained when their unidentified streptomycete culture was grown on a synthetic medium. A British patent claims that increased yields of cobamides were obtained when continuous additions of nutrients were made to hold the reducing sugar level of the medium at 0.01 to 0.05 per cent (79). Glucose, maltose, lactose, sucrose, sorbitol and/or protein hydrolyzates were used as feeds.

Makarevich *et al.* recently reported their studies on the effect of aeration conditions on the production of cobamides by *Propionibacterium shermanii* and *Actinomyces olivaceus* (80). They observed that changes in aeration did not appreciably affect cobamide biosynthesis by *P. shermanii*; however, aeration had considerable effect on the biosynthesis by *A. olivaceus*. With this organism increased aeration stimulated mycelium production of cobamides and it influenced the type of vitamin produced. High aeration in a corn

extract medium caused production of 5,6-dimethyl- α -benzimidazolylcobamide while suboptimal aeration rates on this medium caused production of appreciable quantities of α -adenylcobamide.

A study of the utilization of cobalt by *S. griseus* has been made by Perlman & O'Brien (81). At cobalt levels of less than 1 p.p.m., 75 per cent of the added cobalt was incorporated into the formed cobamide. Many organic and inorganic forms of cobalt were incorporated into the vitamin molecule. An organic cobalt complex formed in a yeast grown on a cobalt-containing medium was found to be more efficiently converted to cobamide than was the cobalt of $\text{Co}(\text{NO}_3)_2$.

Precursors that have been shown to be incorporated into or to stimulate production of cobamides were discussed by Perlman (72). A recent announcement of the commercial production of cobamides from sewage sludge has been received with considerable interest (82).

Riboflavin.—Riboflavin is produced by a number of microorganisms. However, in commercial production *Eremothecium ashbyii*, *Ashbya gossypii*, *Candida* spp., and *Clostridium* spp. are most important. Reviews on the production of riboflavin have appeared in Underkofler & Hickey (3) and in Prescott & Dunn (4). A very comprehensive literature review of flavinogenic organisms, factors controlling flavinogenesis, and the mechanism of riboflavin biosynthesis was prepared by Goodwin (83).

There have been very few publications in recent years on riboflavin production by *A. gossypii*. Riboflavin yields of 2000 $\mu\text{g.}$ per ml. were obtained from a relatively carbohydrate-free medium. The main carbon source was corn oil. It was interesting to find that with the lipid medium, plant protein sources which are normally non-flavinogenic, became flavinogenic (84). Propionate also has been shown to stimulate riboflavin production when it is added to a medium composed of plant protein materials (85).

There has been considerable study on the production of riboflavin by *E. ashbyii*. Goodwin listed ten different natural materials from which the organism has produced significant yields of riboflavin (83). Although sucrose, glucose, maltose, and lard oil were the preferred carbon sources, a great variety of nitrogen sources could be used. Goodwin & Pendlington studied the effects on flavinogenesis of separate additions of either purines, pyrimidines, or amino acids to a chemically defined medium supplemented with peptone (86). Riboflavin synthesis but not cell growth was stimulated by the addition of L-threonine, L-serine and, to a lesser extent, by L-tyrosine. L-Glutamate, L-aspartate, and L-asparagine stimulated both riboflavin production and growth equally; L-cysteine inhibited growth completely. Xanthine and adenine stimulated riboflavin synthesis; however, additions of pyrimidines were without effect. In an extension of these studies, Brown *et al.* observed that purines as stimulators of riboflavin synthesis fell into the following order of decreasing effectiveness: guanine, xanthine, adenine, hypoxanthine, uric acid. The possible mechanisms for conversion of the exogenous purines into riboflavin were discussed (87).

β -Carotene. There has been considerable interest in the fermentation pro-

duction of β -carotene in the last four years. In 1956 Barnett, Lilly & Krause (88) reported they were able to obtain 15 to 20 times more β -carotene per gm. of mycelium from *Choeneophora cucurbitarium* grown in mixed (+) and (-) cultures than when either sex was grown alone. A β -carotene yield of 920 μ g. per gm. of mycelium was obtained from the mixed (\pm) culture. Over the following three years, Anderson and his co-workers of the Peoria laboratories carried on an extensive program on the production of carotenoids with the (+) and (-) mating types of *Blakeslea trispora*. They found that carotene yields were considerably enhanced by the addition of natural oils, white grease, α - and β -ionone, and certain non-ionic surface-active agents (89). Peak yields of β -carotene of over 9000 μ g. per gm. of dry weight were obtained. Studies on the biosynthesis of β -carotene by *Phycomyces blakesleeanus* have been reviewed by Goodwin (90).

. STEROIDS

The steroid hormone sales by United States pharmaceutical firms in 1958 were 120 million dollars (91). Of that total, 95 million were for corticosteroids for the treatment of arthritis and inflammation, and the remainder for sex hormones and progestational agents.

In contrast to most other fermentation products, numerous reviews have appeared on the microbiological transformation of steroids and their application to synthesis of hormones. The reviews by Fried *et al.* (92), Wettstein (93), Shull (94), Eppstein *et al.* (95), Peterson (96), and Vischer & Wettstein (97) deserve special mention. The third edition of Prescott & Dunn (4) contains a number of well-organized figures and tables which were prepared by the authors to demonstrate the versatility of microorganisms in accomplishing steroid transformations.

A timely survey on the status of the production of steroids was prepared by Applezweig (91). In the report the uses, markets, products, production routes, producers, and the future of steroids were aptly discussed. A flow chart listed the raw materials, the intermediates, and the finished products of eighteen different steroid manufacturers in Europe and America.

A most important microbiological transformation in steroids from an industrial viewpoint was the introduction of a hydroxyl group at carbon 11 of progesterone. This transformation was first reported by Peterson & Murray in 1952 (98), and it led to economical production of cortisone and its derivatives from 11 α -hydroxyprogesterone. It was soon found that many organisms contained 11 α -hydroxylases. Dulaney and his colleagues found that the transformation of progesterone to 11 α -hydroxyprogesterone was quite common in the genus *Aspergillus* but only 3 of the 476 *Penicillium* cultures were reported as accomplishing the bioconversion (99). Asai *et al.* surveyed 284 strains of *Rhizopus* sp. and reported that 21 of them oxidized progesterone to 11 α -hydroxyprogesterone (100). The first report of 11 α -hydroxylation of progesterone by bacteria has been given by McAleer *et al.* (101).

The microbiological 11 β -hydroxylation of 11-deoxy-17 α -hydrocortisone

(Compound S) yields hydrocortisone (Compound F). Although a number of microorganisms carry out the transformation (97), *Cunninghamella blakesleeana* (102) and *Curvularia lunata* (103, 104) appear to be the most efficient.

One of the most important microbiological dehydrogenation reactions is the introduction of the 1,2-double bond into ring A of the nucleus to produce 1-dehydrosteroids. In 1955 Vischer *et al.* described the production of prednisone from cortisone by *Fusarium solani* (105), and in the same year Nobile *et al.* showed that it was possible to produce prednisone from cortisone through the use of *Corynebacterium simplex* (106). These 1-dehydrosteroids were more potent in the treatment of arthritis than the parent compounds and they possessed the advantage of having lower salt retention effects. In a recent note Takeda and co-workers (107) reported they were able to 1-dehydrogenate and 11 β -hydroxylate Compound S to prednisolone simultaneously by use of a mutant of a *Pseudomonas* sp. Twenty to thirty per cent of the Compound S was converted to prednisolone.

In recent years a number of modifications of the cortical hormones have been studied in an effort to obtain clinically useful steroids that would have fewer side-effects or be more specific in their action. Spero and his co-workers at Upjohn described a procedure for the production of 6 α -methylprednisolone where the 1,2-double bond was introduced by *Septomyxa affinis* (108). Another commercially important steroid that is obtained in part through a microbiological dehydrogenation step has been reported by Bernstein *et al.* (109). They obtained 9 α -fluoro-16 α -hydroxyprednisolone by introducing the 1,2-double bond into 9 α -fluoro-16 α -hydrocortisol through the use of *Corynebacterium simplex*.

Although a wide range of microorganisms has been shown to be capable of carrying out the 1-dehydrogenation of Δ^4 -3 ketosteroids (10), Meeks *et al.* reported that *Septomyxa affinis* carried out dehydrogenation at carbon atoms 1 and 2 but not at 4 and 5 (110).

The 16 α -hydroxylation of 9 α -fluoroprednisolone recently has been reported by Thoma *et al.* of Squibb (111). The hydroxylation was achieved by the use of *Streptomyces roseochromogenus*. The introduction of the 16 α -hydroxyl group into steroids has increased the anti-inflammatory action of the steroid and reduced the salt retention effects.

There have been a number of papers in recent years on the biochemical aspects of the microbiological steroid transformations. Hayano and co-workers (112, 113) have shown that the enzymatic hydroxylations of the 11 α , 11 β , and 12 β positions of steroid substrates occurred by way of a simple replacement of the hydrogen of the position subsequently oxygenated. In 1956 Marcus & Talalay obtained and purified two adaptive hydroxysteroid dehydrogenases from cell-free extracts of *Pseudomonas testosteroni* (114). A year later Perlman *et al.* reported that steroid oxidation systems of two *Streptomyces* species were adaptive in origin (115). Further evidence of the adaptive nature of the enzymes was obtained when it was found that antibiotics inhibited formation of the steroid-oxidizing system. Dulaney and his

colleagues concluded that the inability of zinc-deficient cells of *Aspergillus ochraceus* to 6 β -hydroxylate 11 α -hydroxyprogesterone was caused by the lack of the required adaptive enzyme (116).

The steroid transforming ability of a number of microorganisms has been shown to be changed by mutation (104, 107), and Dulaney was issued a patent which claimed that through a process of strain selection on specific media it was possible to obtain *Saccharomyces cerevisiae* strains that contained as much as 11 per cent ergosterol (117). This sterol may be used as a starting material for steroids.

One of the few publications concerned with the effect of physical variables on microbiological transformations in large scale equipment was reported by Karow & Petsiavas (118). The rate of hydroxylation of specific steroids by *A. ochraceus* was greatly dependent on mechanical agitation and air flow. They also observed that semicontinuous addition of substrates was superior to batch additions because it reduced solvent toxicity, permitted higher steroid concentrations, decreased mechanical loss of the substrate, prevented secondary transformations, and provided for more accurate timing in the termination of the bioconversion.

Vischer & Wettstein (97) and Talalay (119) have discussed the many facets of the enzymes concerned with microbiological transformations and steroid metabolism. Wallen, Stodola & Jackson recently completed a systematic survey of the literature on type reactions in fermentation chemistry (120). Included in their survey were almost 500 examples of microbiological transformations of steroids.

ORGANIC ACIDS

Citric acid.—Increased uses for citric acid and its derivatives have stepped up interest in the fermentation. Aside from the normal medicinal and food industry uses, increased fermentation production has been necessitated because of the use of the product in plasticisers and as an iron sequestering agent to assist in secondary oil recovery. Recent developments in the citric acid fermentation include the change from the older established shallow-pan method to a deep-tank submerged method. Reviews of the citric acid fermentation have appeared in Underkofler & Hickey (2) and in Prescott & Dunn (4).

A group headed by S. M. Martin of the National Research Laboratories of Canada has been especially active in the development of the submerged production of citric acid from ferrocyanide-treated beet molasses by *Aspergillus niger*. Factors studied were effects produced by changes in sterilization technique, initial pH, inoculum, inoculation rate, ferrocyanide treatment of molasses, phosphate levels, fermentation temperature, aeration rate, and scale-up. The basic conclusions from these studies were summarized in a paper by Martin (121). They obtained an average yield of 8.2 per cent anhydrous citric acid which corresponded to 68.5 per cent conversion of the available sugar. The fermentation time varied considerably with the batch

of molasses used. Aeration was the main problem in the scale-up and in this regard Murphy, Clark & Lentz recently have reported on studies of physical factors which affected aeration in their tower-type fermentor (122). Aeration methods for submerged production of citric acid have been studied by Berk & Brauerman (123). They obtained 10.5 per cent citric acid, which corresponded to a conversion of 93.5 per cent of the sugar added, when they aerated their fermenting medium with a special vibro-mixer. In 1959 two American companies announced plans to begin submerged production of citric acid (124). It is rather surprising that the production of citric acid by a continuous process has not been reported.

To obtain high yields of citric acid from cheap crude carbohydrate sources, certain of the minerals which are essential for cell growth must be removed at considerable expense. James *et al.* described a procedure in which it was possible to obtain *A. niger* mutants which were more metal-tolerant than was the parent (125). The mutants produced citric acid at a yield equivalent to 80 per cent of the sugar fermented.

In Russia, Imshenetskii *et al.*, through the use of ultraviolet radiation, obtained an *A. niger* mutant that produced 16 to 22 per cent more citric acid than the parent strain which was used in commercial production of this product (126). The mutant strain produced 25 to 30 per cent less mycelium than the parent yet it consumed 26 to 51 per cent more sucrose per gram of dry mycelium. The yield of citric acid from the sugar consumed varied from 57 to 74 per cent.

Acetic acid.—Very little work has been reported in recent years on what is probably one of the oldest of all applied fermentations, production of acetic acid from ethyl alcohol. Most of the advances made in the vinegar fermentation since Pasteur's time have been mechanical rather than microbiological. Two reviews that discuss the over-all fermentation have appeared in Underkofler & Hickey (2) and Prescott & Dunn (4). Rao prepared a comprehensive literature review on acetic acid bacteria (127).

Hromatka & Ebner recently described the equipment, operation requirements, and yield calculations concerned with the use of the "Acetator" for the production of vinegar by submerged oxidative fermentation (128). The vinegar produced by this method was claimed to be equivalent to or better in flavor and aroma than vinegar produced by older methods and the yield was higher.

Lactic acid.—The annual fermentation production of lactic acid continues to increase. Kempe *et al.* at the University of Michigan have used a steady-state lactic acid fermentation to determine temperature coefficients and activation energies (129). By the combining of low initial nitrilite concentrations with continuous addition of the nitrilites it was possible to obtain in steady-state a high over-all rate of lactic acid production. From such fermentations a Q_{10} value of 2.5 between 30° and 40°C. and an activation energy of 17,100 calories per gm. mole were calculated.

Other acids.—Literature on the production of a number of organic acids

including ustilagic, kojic, itaconic, gluconic, fumaric, and the keto acids has appeared in the last six years. The fermentation production of these acids will not be reviewed here. Pertinent references to these fermentations may be found in Underkoffler & Hickey (2, 3) or in Prescott & Dunn (4).

ALCOHOLS AND SOLVENTS

Acetone, butanol.—In recent years the fermentation production of acetone, butanol, and ethanol has operated on a marginal basis in the United States and Great Britain. The decrease has been brought about by increased production from petrochemicals and by the increasing costs of molasses. In Japan and India where synthetic processes are not extensive, fermentation remains the major method of production. Three extensive reviews of the acetone-butanol fermentation have appeared (2, 3, 33), and Barker (130) has reviewed thoroughly the chemistry of the butyric acid-butanol fermentations.

Many of the recent studies on the acetone-butanol fermentation have attempted to replace part or all of the molasses of the media with cheaper, more available or more productive energy sources. Nakhmanovich & Shcheblykina have shown that the mixture of pentoses obtained from acid hydrolysis of corn cobs can partially replace the wheat and maize flour used for acetone-butanol production in the U.S.S.R. (131). The addition of either calcium acetate or acetic acid to an acetone-butanol fermentation has been shown to increase the solvent yield to 40 per cent based on the sugar utilized (132). Two publications have appeared on the production of acetone-butanol by continuous fermentation. Finn & Nowrey observed that they obtained less strain degeneration when *Clostridium saccharoacetobutylicum* was maintained in logarithmic growth in a continuous propagator than when the same strain was serially transferred in flasks every 24 hr. (133). They emphasized the point that broad generalizations about culture stability should be interpreted cautiously. Dyr *et al.* described conditions for continuous fermentation production of acetone-butanol (134). They obtained solvent yields of 32 per cent in their batch fermentation and in their continuous fermentation. However, the batch method was complete in 100 hr.; the continuous method had a hold-up time of only 30 hr.

Ethanol.—There have been few recent papers on the fermentation production of alcohol. Excellent reviews of the alcoholic fermentation of grain, molasses, sulfite waste liquor, and wood waste have appeared in Underkoffler & Hickey (2), and Prescott & Dunn have reviewed the literature on the production and the mechanism of the ethanol fermentation (4).

A report on a pilot-scale evaluation of a submerged fungal amylase process for grain alcohol fermentation was prepared by Hanson *et al.* (135). The process was found practical and economically feasible, the yield and quality of neutral spirits was equivalent to that obtained from the malt process, and the yield of distillers solubles was slightly higher. Corn and wheat containing up to 50 per cent of damaged kernels could be used efficiently. A possible

disadvantage was the increased alcohol fermentation time of the fungal amylase method. A pilot-scale study of the continuous alcoholic fermentation of blackstrap molasses by *Saccharomyces cerevisiae* was made by Borzani & Aquarone (136). Agitation was considered to be the rate-determining factor for the fermentation. An increase in the alcohol yield was observed when one unit of penicillin per ml. was added to the fermentations. The added penicillin did not affect the final yeast count or the fermentation time.

Glycerol.—Glycerol is another of the fermentation products which has received vigorous competition from the petrochemical field. The biological production of glycerol has been discussed in detail (2). In the majority of the processes a yeast fermentation was "steered" by sulfite or alkali to produce the glycerol. In a series of three papers Freeman & Donald (137) described the effects of many variables associated with the anaerobic production of glycerol in pilot-scale equipment. The addition of sulfite was shown to be an important factor in determining the relative yields of glycerol, ethanol, acetaldehyde, and acetic acid. It was not possible to acclimatize yeast strains to ferment glucose in the presence of high levels of sulfite-bisulfite. Glycerol yields to 24 per cent (based on sugar utilized) were obtained when a distillers yeast strain of *S. cerevisiae* was grown in medium which contained blackstrap molasses and additions of Na_2CO_3 equivalent to 30 per cent of the fermentable sugar of the medium.

Spencer & Shu found it was possible to produce glycerol with osmophilic yeasts without the use of steering agents. In one of their later papers on the production of glycerol by *Saccharomyces rouxii* P_{3a} it was observed that when oxygen tension was increased yields of glycerol and arabitol increased and those of ethanol decreased (138). When inorganic phosphate levels were increased the opposite effect on yields was obtained. Peterson *et al.* studied factors affecting production of polyhydric alcohols by a number of *Zygosaccharomyces* species (139). Three of the strains produced 35 to 45 per cent glycerol in a 10 per cent glucose fermentation. Aeration was an important factor in increasing the yield of the glycerol and arabitol but the glucose concentration of the medium modified the air requirements.

AMINO ACIDS

There has been considerable interest in the fermentative production of amino acids in the past three years. At least two amino acids, L-lysine and L-glutamic acid, are being produced on a commercial scale and it is likely that a number of the others will be produced by fermentation methods should markets develop. A comprehensive survey of the microbiological production of amino acids has been prepared by Kinoshita (140) and Kita (141).

Glutamic acid.—In 1957 Kita, in an issued patent, described the production of 2 to 3 gm. glutamic acid per l. from a filtered broth when a number of *Cephalosporium* species were inoculated to a variety of media composed of a number of natural nitrogen sources (142). At about the same time, Asai *et al.* reported on the production of L-glutamic acid from a chemically defined

medium by *Micrococcus varians* (143). Kinoshita and his co-workers obtained a number of aerobic, non-sporulating, Gram-positive biotin-requiring cocci which were capable of producing over 30 gm. of glutamic acid per l. from a glucose and ammonium salts or urea medium. The organism was called *Micrococcus glutamicus* (140, 144, 145).

Lysine.—The fermentative production of lysine has been described in patents issued to Casida (146) and to Kita & Huang (147). In the Casida patent a mutant *Escherichia coli* strain grown on a glycerol-corn steep liquor-salts medium produced diaminopimelic acid. Lysine needed for growth of the organism was obtained from the corn steep liquor. In the next step, non-lysine-requiring strains of *E. coli* or *Aerobacter aerogenes* were grown and the enzyme diaminopimelic acid decarboxylase was produced. The cells were ruptured with toluene and when the broths were mixed the diaminopimelic acid of the first fermentation was converted to L-lysine by the enzyme produced in the second fermentation. In the Kita and Huang patent, a lysine-requiring strain of *E. coli* was grown under aerobic conditions and after growth the cells were ruptured and the production of L-lysine was then accomplished under anaerobic conditions.

Kinoshita obtained 20 gm. L-lysine per l. when a homoserine-less mutant of *M. glutamicus* was inoculated into a glucose-natural protein-salts medium (148) and Richards & Haskins (149) screened over 600 strains of fungi for production of lysine in a chemically defined medium. Twelve of the strains produced 5 to 15 gm. lysine per l. of culture filtrate.

Other amino acids.—The production of high yields of L-ornithine by a *Micrococcus* mutant has been reported by Kinoshita (145) and workers at Eli Lilly & Co. have described pilot studies on the production of L-tryptophan (150).

MICROBIAL ENZYMES

Prior to 1950 most commercial microbial enzymes were produced by surface culture methods. In recent years submerged culture methods have come into extensive use. The major uses of microbial enzymes are found in the food, pharmaceutical, textile, paper, and leather industries. Most of the commercially-important microbial enzymes catalyze the hydrolysis of natural organic compounds. Glucose oxidase and catalase are, however, non-hydrolytic.

Two comprehensive reviews on the production of microbial enzymes appeared in 1954. Underkofler (3) discussed the nature, production, and application of fungal amylolytic enzymes. The major potential use for the fungal amylases was as saccharifying agent for grain alcohol fermentation mashes. Most of these enzymes were obtained from strains of *Aspergillus* or *Rhizopus* species. Hoogerheide [in (3)] reviewed the uses and industrial production of bacterial amylases, bacterial and fungal proteases, pectolytic enzymes, and invertase. Four other commercial enzyme preparations were marketed at that time: catalase, glucose-oxidase, penicillinase, and streptokinase-streptodornase.

A comparison of the surface and submerged processes for the production of microbial enzymes was discussed by Underkofler *et al.* in a recent paper (151). A detailed discussion of the applications of microbial enzymes also was presented. Scott surveyed the actual and potential applications of catalase (152) and was issued a patent on the use of the enzyme for a deoxygenation packet (153). The packet is a film of polyethylene which contains glucose oxidase-catalase, glucose, and buffer. When the packet is placed in a sealed container the residual oxygen is immediately destroyed and an oxygen-free atmosphere is obtained. Use of the packet would be of great value in extending the shelf life of packaged foods which ordinarily deteriorate due to rancidity or oxidative changes.

Clinical use of enzymes has developed at a rapid rate in recent years (154). Microbial proteases have been used for digestive aids, debridement of wounds and for relief of inflammation, bruises, and blood clots. The presence of glucose in urine may be detected by a simple strip of filter paper which is impregnated with glucose oxidase, peroxidase, and an indicator (155). Increased clinical use of microbial enzymes can be expected as more fundamental knowledge is obtained about the physical ailments that result from disturbed metabolic enzyme systems.

GIBBERELLINS

The gibberellins are a group of closely related plant growth-promoting metabolites of *Gibberella fugikuroi* (conidial state: *Fusarium moniliforme*). Stodola has prepared a comprehensive literature survey on gibberellin (156) and Brian & Grove have written a review on the production, physiology, and chemistry of gibberellic acid (157).

Kitamura *et al.*, who described the first submerged fermentation for production of gibberellins in 1953 obtained yields of 7 to 8 mg. per l. (158). Two years later Stodola and his associates described fermentations in pilot equipment in which they obtained yields of gibberellins A and X totalling 22 mg. per l. (159). The yields were obtained when *F. moniliforme* NRRL 2284 was grown on a 2.5 per cent glucose and salts medium for 3 days.

In 1955 Borrow *et al.* described a fermentation with *F. moniliforme* Kew 917 from which they obtained yields of gibberellic acid of 180 mg. per l. in 18 days (160). They used a 4 per cent glucose and salts medium. Recently Darken, Jensen & Shu described fermentations with the Kew 917 strain with which they obtained gibberellic acid yields of 880 mg. per l. in shaken flasks and 650 mg. per l. in 1000 gal. fermentors. The high yields were made possible by the combined use of corn steep liquor and such slowly-utilized carbon sources as glycerol and lactose (161). The potential of gibberellic acid in agricultural practice has yet to be developed. At present its use is restricted to specialized greenhouse applications.

MICROBIAL POLYSACCHARIDES

The commercial production of dextran as a blood plasma extender has virtually ceased in the United States. However, limited production may occur

in other countries. From 1950 to 1955 a group at Peoria was actively engaged in studies of the enzymatic synthesis of dextran by *Leuconostoc mesenteroides* NRRL B-512 (162, 163). They observed that the molecular weight of the dextran was affected by the enzyme concentration, sucrose concentration of the medium, primer type, primer concentration, and the reaction temperature. Shinoda (164) recently described a procedure for the isolation of strains of *L. mesenteroides* which produced high yields of dextran. Also described were studies designed to determine the optimum conditions needed for dextran formation in shaken flasks and in pilot tanks.

New uses for dextran other than as a plasma extender have been described (4, 10, 11, 12). A number of laboratories are investigating the microbial production of polymers with specific properties.

FOODS AND FEEDS

There have been a number of interesting reports on the potential applications of microorganisms for the production of food. The papers written by Thatcher (165), Dunn (166), Woodbine (167), Anderson & Jackson (168), and the two papers by Reusser *et al.* (169) are especially worthy of mention.

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INTRACELLULAR SYMBIOSIS IN INSECTS¹

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"By endosymbiosis we understand a regulated, harmonious cohabitation of two nonrelated partners, in which one of them lives in the body of the other usually more highly organized being, and in which the mutual adaptation has reached such a high degree of intimacy, that the supposition is justified, it could be a useful arrangement for the host." This is the modern concept of symbiosis as defined by Paul Buchner, the pioneer of symbiosis research, in the latest edition of his book, *Endosymbiose der Tiere mit pflanzlichen Mikroorganismen* (5).

The botanist, de Bary, gave symbiosis a much broader definition (16). In 1879 at the congress of natural science in Kassel he coined the term, "symbiosis," and defined it by using the lichen as an example. His definition included "antagonistic symbiosis," which is better considered as "parasitism in opposition to mutual symbiosis." Nevertheless, there are no sharp boundaries between the two categories and many cases of cohabitation of non-related partners lead from one to the other.

In our discussion we will limit ourselves solely to "endosymbiosis" and omit all other forms of mutual symbioses; lichen, mycorrhiza, leguminous root-nodules, etc., will not be considered here.

We are indebted chiefly to Paul Buchner and his school for the abundance of material which has been gathered in the fifty years of rapidly developing symbiosis research. The results are so comprehensive that today we are quite well informed as to the boundaries of the symbiosis principle and in this field we can expect nothing essentially new to be discovered. At the present time, the emphasis in symbiosis is being placed on the explanation of the physiological mutual relationship between the animal host and its guests, the plant organisms. It is necessary to be well oriented on this point, if one wishes to discuss the meaning of this cohabitation.

The very presence of such symbioses permits certain assumptions. All insects that suck plant sap, those that feed on vertebrate blood for their entire life span, and those that eat wood and ceratin, have symbionts. They are all one-sided specialists in nourishment. With these may be classified a few insects that are harmful to stores and provisions, such as the *Calandra* (*Sitophilus*) species, the drugstore weevil *Sitodrepa panicea*, *Lasioderma sericorne*, and *Rhizopertha dominica*, which stem from wood-eating ancestors and have adapted themselves only secondarily to this way of life. Also, a few omnivorous insects, such as cockroaches and the Australian mastoter-

¹ The survey of the literature pertaining to this review was concluded in December, 1959.

² Dedicated to Prof. Dr. Otto Koehler, Freiburg/Breisgau, on the occasion of his seventieth birthday.

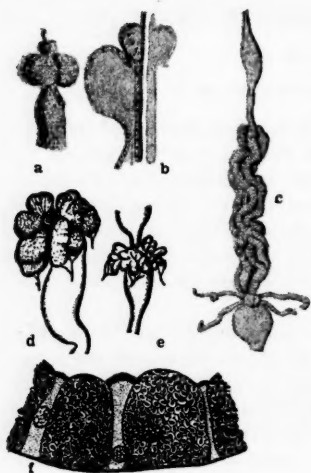


Fig. 1

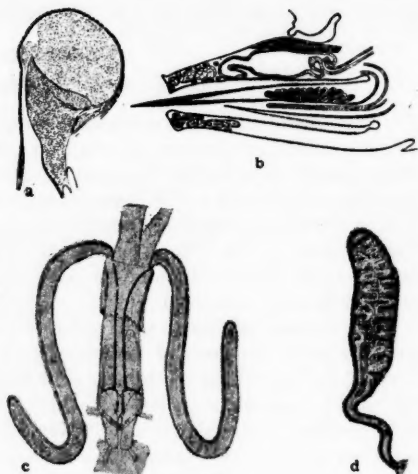


Fig. 2

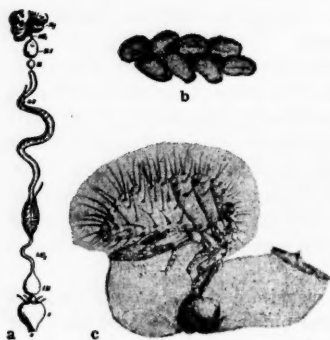


Fig. 3

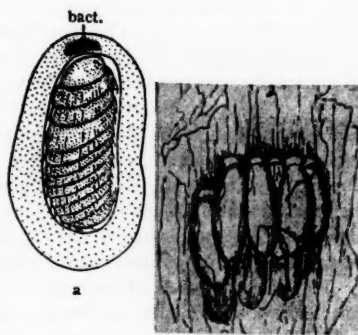


Fig. 4

PLATE I

FIG. 1. Intestinal symbiosis. a, b: *Dacus oleae* Gmelin. Blind sacks of the mid-gut, the lumen of which is filled with symbiotic bacteria (after Petri). c: *Carpocoris fuscispinus* Boh. Intestinal canal of adult with four rows of crypts filled with bacteria (after Kuskop). d, e, f: *Sitodrepa panicea* L. Blind sacks of the mid-gut (d: from a larva; e: from an adult), the epithelium of which is infected with symbiotic yeasts (after Koch). f: Single mycetocytes, between which are epithelial cells of the gut with cilia (after Breitsprecher).

FIG. 2. Arrangements for the smearing of eggs. a: *Sirex gigas* L. Intersegmental

mites, both of which stem from a common ancestor, as well as some species of ants (*Camponotus ligniperda*, *Formica fusca*), are hosts to symbionts. In these cases it is not easy to explain the significance of the cohabitation with microorganisms. A particularly specialized group of animals harbor bacteria or fungi in their organs of excretion (some Apionidae, the Donaciinae, certain ticks (*Rhipicephalus sanguineus*, *Boophilus annulatus*, *Ixodes* species), which make room for their symbionts in the Malpighian tubules; in the case of earthworms and Hirudineae we find them in the segmental organs; and in the Cyclostomatidae, Annulariidae, and Molgulidae they occur in the storage kidneys.

In conclusion, we may name the glowing Teleosts and Cephalopodae, the Pyrosomae and Salpae, in which the deeper significance of cohabitation with light-producing bacteria is much easier to establish.

A common factor of all these symbioses is the strict continuity of the cohabitation from generation to generation. It is but seldom that the re-infection with symbionts is left to chance. This, for example, is the case with many illumination symbioses. The glandularly constructed illuminating organs open direct to the outside (*Monocentris*, *Anomalops*, *Photoblepharon*, *Malacocephalus*, Sepiolidae, etc.), or into the esophagus (*Leiognathus*), with the result that through the abundance of free-living illuminating bacteria in sea water an entrance of the organisms into the glandular tubes is assured.

However, in most cases, the transmission of symbionts takes place by means of the egg, either through infiltration of the cocoon fluid by bacteria within the mother's body (Lumbricidae), or through a more or less heavy smearing of the shell externally. The latter method is chosen by insects with intestinal symbionts without exception, whether the microorganisms inhabit the lumen of the gut (Trypetidae, Heteropterae) or live intracellularly in the gut epithelium (Anobiidae, Cerambycidae, Cleonidae, Donaciidae (*Cassida*, *Bromius*)) (Fig. 1, Plate I).

For the infection of eggs, reservoirs of symbionts are located in a suitable

pockets (longitudinal section) with oidia of the symbiotic fungus; to the side the smearing gland (after Buchner). b: *Dacus oleae* Gmelin. Longitudinal section through the laying apparatus, with the smearing arrangement brought to bear on the terminal-gut (after Petri). c: *Anobium striatum* Oliv. Laying apparatus with intersegmental tubes and vaginal pockets (after Breitsprecher). d: *Cleonus piger*. Scop. Bacterial syringe in longitudinal section (after Buchner).

FIG. 3. *Coptosoma scutellatum* Geoffr. a: Female gut (Mg, stomach; MD₁, short section of mid-gut; BLS, blind sack; BL, small, bladder-shaped section; KrD, cryptic-gut; MD₂, tubular-shaped section; EBL, terminal bladder; R, rectum). b: Eggs with symbiotic capsules. c: newly hatched larvae sucking at the symbiotic capsules (a: after Schneider; b,c: after H. J. Müller).

FIG. 4. a: Infectious mass of bacteria in the gelatinous capsule of an egg of *Donacia*. b: Eggs of *Cassida viridis* L. Each egg has a bacterial cap (after Stammer).

part of the female sexual tract and act at the same time as smearing mechanisms. These are developed into various forms such as syringes, sacks, or pockets in the Siricidae, Anobiidae, Cerambycidae, Cleonidae, Lagriidae, Trypetidae, and in some heteropterous bugs (Fig. 2, Plate I).

In the case of bugs we can recognize a gradual development from very primitive arrangements to the most complicated mechanisms. Use is made of the following possibilities: In the blood-sucking Triatomidae (*Rhodnius prolixus*), the symbionts are located quite far up in the mid-gut, nevertheless, a suitable number of bacteria are carried with the feces into the terminal-gut and from there to the outside, with the result that the eggs, in the laying process, are soiled with the fecal bacteria. The infection of the hatching larvae is secondarily assured, in that the young larvae, accustomed to sucking on the fecal droplets of their older companions, ingest the bacteria. Such instincts, which force the newly hatched larvae to the source of infection, are also known in other bugs. In *Brachypelta aterima*, a Cydnid which digs in sand, the symbionts are located in the cryptic-gut, which is characteristic of bugs and is located in the most posterior part of the mid-gut. When the female is ripe for the laying of eggs, the crypts swell because of an increase in the number of bacteria therein, and these organisms are expelled with the fecal droplets. This explains the following brooding habit of these bugs. The female remains by its eggs until the larvae hatch, and the latter stay about one week with the mother. During this period they suck at the fecal droplets, filled with symbionts, which leave the anus of the female. In this manner they take up their symbionts.

Even this case of the joining of instinctive action with the demands of symbiotic cohabitation is excelled by a representative of the Plataspidae. The female of the species *Coptosoma scutellatum* lays down a fairly large, brown-red capsule in regular sequence between every two of her eggs, which are deposited in rows (Fig. 3b, Plate I). These capsules are formed in the enlarged, club-shaped, terminal part of the mid-gut and are filled with a pure culture of symbionts. The symbionts are cultivated as specially modified infectious forms in the bulbously enlarged terminal section of the cryptic-gut, which is lacking in the male (Fig. 3a, Plate I). The larvae hatch from the eggs, which have lids, and after a short period of rest following hatching, they pierce the symbiotic capsules and sip out their contents. Only then do they leave the area and its empty shells (Fig. 3c, Plate I).

In the Acanthosominae still another possibility of transmission is realized, which is reminiscent of corresponding arrangements in the Anobiidae. Here both cryptic rows have become completely independent and have lost their connection with the intestinal tract. Since transmission by way of the gut is no longer possible, a new means must be found. There resides in the female, in the ventral, posterior end of the body, a special symbiont reservoir, which lies in the region of the egg-laying apparatus and likewise serves as the organ of transmission. It is pear-shaped and its chitinous coat forms a system of

delicate, parallel tubules that contain bacteria for the smearing of the eggs.

The Chrysomelidae (*Cassida viridis*) solve the problem of the infection of the eggs in the following way: in the act of egg-depositing, a cap containing bacteria is placed upon the upper pole of the egg, where the anterior end of the larva is destined to lie (Fig. 4b, Plate I). The Donaciinae behave in a similar manner. They deposit a mass of bacteria at the same spot on the gelatinous egg covering (Fig. 4, Plate I).

However, in all cases where the symbionts are housed in the region of the body cavity, entirely different means of transmission must be chosen. The infection must take place much earlier in the mother's body and, in extreme cases, as in *Calandra* (*Sitophilus*) for example, the primary germ cells are provided with the symbiotic inheritance (Fig. 5a, Plate II).

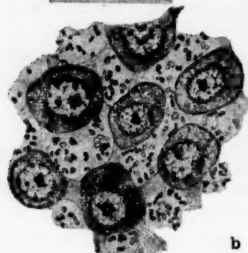
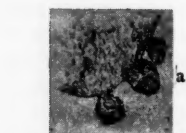
Entry into the egg is not always the same, and very different solutions attain the same result. The symbionts of the Lyctidae and the closely related Bostrychidae penetrate the egg from all sides. This, however, is possible only during a very short period of time. Immediately after the cessation of egg growth, the follicular cells spread out and build a loose network that opens up the path into the inside of the egg through wide gaps (Fig. 5b, Plate II). These portals then close and the secretion of the chorion prevents further entry.

The ants (*Camponotus*, *Formica*) are another example of an apolar entry of the symbionts into the egg. In this case the follicular cells are infected quite early and the entry of the bacteria into the growing egg results from within. The bacteria grow rapidly and finally fill the entire plasma of the still young egg (Fig. 5c, Plate II).

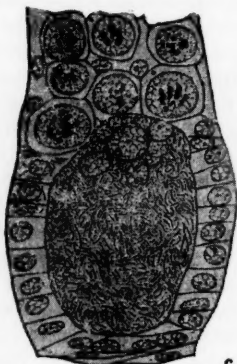
Much more frequent, however, is the polar entry of the symbiotic organisms, over either the anterior or the posterior pole of the egg (14). In the former case, as, for example, in many Coccidae [*Lecanium*, *Pseudococcus*, *Macrocerococcus* (13), etc.], the Curculionidae (with the exception of the Cleonide), certain heteropterous bugs (insofar as they possess mycetomes), and all the Ipidae which have been investigated thus far, the symbionts pass between the follicular cells into the plasma of the egg, or they drift with the secretion of the nourishing cells into the egg (Curculionidae, Ipidae, Heteropterae) (Fig. 6, Plate II).

The other possible entrance at the lower pole allows a choice between an intrafollicular infiltration and the temporary infection of special follicular cells ("wedge cells"). Fig. 7a, Plate II which can easily be recognized as such preferred elements by their large size and their circular arrangement.

So far, bipolar infiltration has been observed only in the polysymbiotic crickets. In *Enchophyllum 5-maculatum*, for example, which possesses six different types of symbionts, two rod-shaped species of bacteria enter the egg from above, over the nourishing cord. This sharply delineated symbiotic bundle lies in the ripe egg at the posterior pole, to which the remaining four symbionts give a colorful, checkered appearance (Fig. 7b, Plate II). Special



b
Fig. 5



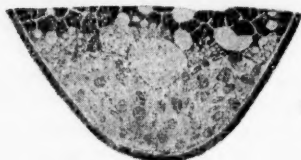
c



Fig. 6



a



b

Fig. 7

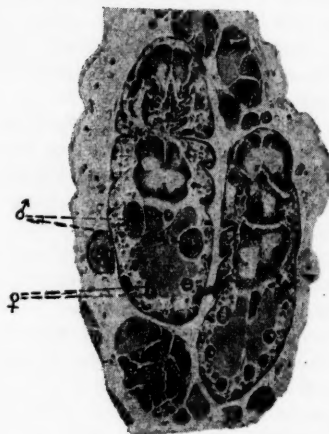
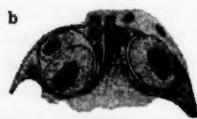


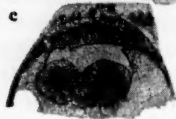
Fig. 8



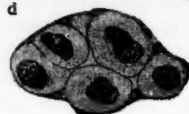
a



b



c



d

Fig. 9



Fig. 10

ovarian mycetomes, in a suitable location, often deliver the easily transportable transmission forms which, in many crickets, are cultivated in separate compartments of the mycetome, the infection mounds (the ovarian ampules of the Pediculidae and the Mellophagae, the transitory ovarian mycetome of *Marchalina*, the ring-shaped filial mycetome of the bugs *Nysius* and *Ischnorrhynchus*, the ovarian mycetome of *Fulgora confusa*).

Without doubt, the majority of symbionts were first taken up with nourishment by way of the mouth. Thus, it is understandable that the foreign guests first settled in the lumen of the gut and from there penetrated deeper into the body. In many insects (Lamellicornia, Tipulidae, certain termites), voluminous nourishing chambers have been set up which serve for the microbial utilization of cellulose, as is also the case for the first stomach of ruminants, the cecum of the horse, and the appendix of the rodent. Alternatively, pouches are set up in the region of the anterior mid-gut for the taking up of symbionts, and they are more or less closed off from the gut (*Dacus* and related Trypetinae) (Fig. 1a, b, Plate I). In the cryptic-gut of heteropterous bugs it is a case of garland-shaped, wound rows of hundreds of diverticula in the most posterior part of the mid-gut, and their lumina are filled with pure cultures of symbionts (Fig. 1c, Plate I).

Fig. 5. Different modes of infection. a: *Calandra granaria* L. The bacteria are migrating into the primary germ cells. b: *Lyctus linearis* Goeze. Two different types of symbionts wander through gaps in the follicular epithelium into the egg. c: *Campenotus ligniperda* Latr. Infection of the follicle and the young ovum (a: after Scheinert; b: after Koch; c: after Buchner).

Fig. 6. *Bladina fraterna* Stal. Upper end of an egg tube. In the plasma of the nourishing chamber are a few vacuoles with symbionts, behind which is the ovarian mycetome. The larger nourishing cord at right appears twice in the section and contains numerous symbionts (after H. J. Müller).

Fig. 7. a: *Paramesus nervosus* Fall. Infection of "wedge cells" (after Buchner). b: *Enchophyllum 5-maculatum* Jrm. Posterior end of egg after infection. Four different kinds of symbionts which entered at the posterior end of the egg over the wedge cells; over these is a round, well-defined bundle of bacteria that has migrated here from the upper pole (after Rau).

Fig. 8. *Pemphigus spirothecae* Pass. Frontal section through sexupara embryos from a chief-host virgin. ♂—male embryo; ♀—female embryo (photomicrograph from Lampel).

Fig. 9. *Macrocerococcus surperbus* Leon. Entrance of the secondary mycetocytes into the egg. First the mycetocytes lie against the neckpiece of the ovariole, (a), and after the follicle, (b), they sink into a shallow pit in the upper pole, (c). d: "Somatic fertilization." Each yolk nucleus lies next to a mycetome nucleus with which it later fuses (after Buchner).

Fig. 10. *Hippeococcus wegneri* Reyne. Sterile mycetome forerunner above the germinal strip (after Buchner).

The intimacy of the cohabitation can be increased if the plant guests are taken up by the epithelial cells of the gut. In the Anobiidae and Cerambycidae as well as in the Cleoninae, the larvae possess rather large diverticula of the mid-gut, which are inhabited by yeasts or bacteria. Under the heavy burden of the symbionts reproducing in the cells, the nuclei become enlarged and polyploid in shape and the cells lose their cilia (Fig. 1f, Plate I). In other cases, the symbionts are merely contained in a narrowly circumscribed zone of the mid-gut which can be differentiated from the noninhabited neighboring regions only by its greatly enlarged cells.

It is no wonder that the intestinal symbionts have found their way frequently into the lumen or the cells of the Malpighian tubules. Such an extracellular colonization of the Malpighian tubules is known at present only in the larvae of the Chrysomelida, *Bromius obscurus*, while *Aspidapion* and other Apionidae are characterized by an intracellular invasion of the excretory organs. In *Ixodes hexagonus*, too, the cells of the Malpighian tubules are uniformly filled with thin, thread-like symbionts, while in *Dermacentor* and *Rhipicephalus* only the last fourth are invaded, and in *Boophilus* the button-shaped, thickened, terminal section of the tubules contain symbionts. In some of the Apionidae (*Erythrapiion*, *Protapion*, *Perapion*, *Oxystoma*), two of the six Malpighian tubules undergo a radical change in form and function. They are transformed into club-shaped, thickened mycetomes, which are attached to the gut merely by thin stems, a process which takes place in the same way in *Coccotrypes*, an Ipida.

It is no longer a very long step from the conditions in which the mycetocytes are completely separated from the gut epithelium and are inserted between it and the muscularis (*Camponotus*, adults of *Hylobius*, and *Gymnetron*), to mycetomes which are completely isolated from the gut and are located in the region of the mesoderm. Recalling the details of the embryonic development of many symbiotic hosts will point out how this probably came about. It is not possible in such a limited survey to consider all of the various solutions which have been found for the housing of symbionts in the space between hypodermis and gut. However, it should be mentioned that often mycetomes of considerable size and number have been developed, and that in polysymbiotic forms each type of symbiont is given a special place, either in partial mycetomes (Fulgoridae and others) or in definite sections of mycetomes (Lyctidae, *Trixagus*, many Aphidae, Membracidae) [see Buchner (5), Figs. 172, 177, 187].

Thus far, I have presented a short survey of that factual material which seemed to me to be essential to a better understanding of what is to follow.

Of the latest works on symbiosis, the studies of Buchner on the symbionts of the Coccidae should be given first place. Although this field had already been well investigated by him and his students, his results have brought many surprises.

A coccida, *Stictococcus sjoestedti* Cock., which is found on the cocoa tree

and other vegetation of tropical Africa, lives in symbiosis with thin, rod-shaped microorganisms whose large numbers thoroughly penetrate the mycetocytes (7, 10). These cells, which contain the symbionts, can be traced back to early cleavage cells. They lie thickly congregated in the peripheral regions of the fat body of the female, without a special mycetome being formed. During the growth of the female sexual organs the ovarioles, to some extent, come in direct contact with the mycetocytes. The loosely packed cells which cover the ovarioles and which are poor in plasma content, engulf the neighboring mycetocytes and carry them along in the course of development into the posterior region of the ovariole. The formerly rod-shaped microorganisms develop into the large, oval-shaped infective forms, and enter the oocytes through wide gaps in the follicular epithelium near the posterior end of the egg follicle. Finally, they fill the entire plasma of the egg which contains very little yolk. Quite unique are the transformation of the originally superficial type of cleavage to complete cleavage, the long survival of the regulating bodies which finally enter one of the primary mycetocytes, the strange detours which take place during the development of the embryo, and many other characteristics.

Some of the egg cells, which do not come in contact with the mycetocytes, are not infected. In the case of *Stictococcus diversisetae* Siv., these non-infected eggs give rise only to males which are small and reduced. Otherwise, both sexes in the Coccidae are provided with symbionts, even though the males of *Macrocerococcus* and *Puto* possess markedly degenerated mycetomes.

Such symbiont-free, greatly reduced males were found by Toth in 1937 in *Pemphigus* and *Stomaphis*. Lampel, in 1958 and 1959, worked with the genesis of the male sexualis generation, in connection with a detailed analysis of the symbiotic cycle of all generations of different Pemphiginae (34, 35). According to his reports, eight egg tubes are present in each sexuparaembryo and their terminal compartments lie at the side of and dorsal to the mycetome. The two most anterior tubes undergo an accelerated development and deliver only male sexualis embryos (Fig. 8, Plate II) which are always free of symbionts. However, during their embryonic development, certain characteristics point to the original conditions where symbionts were present.

In the case of *Stictococcus*, the decision whether or not an oocyte is to develop in the male or female direction, is dependent upon the taking-up of symbionts. Buchner could not decide whether this is a case of purely phenotypical determination of sex according to Hartmann's definition, or whether genetic factors play the chief role. On the other hand, in the Pemphiginae the sex determination is definitely diplogenotypical and has nothing to do with the symbionts.

S. diversisetae caused some surprise insofar as yeastlike microorganisms not only colonize the mycetocytes, but also penetrate the body of the host on all sides (8). These microbes are quite similar to the symbionts of the Lecaniae, certain Asterolecaniae, and Tachardinae, and they could be classi-

fied as Ascomycetes. In the symbiotic form they reproduce only as conidia. while in pure culture they grow in mycelium-like, budding clusters.

Buchner assumes that the ancestors of *S. diversisetae* had a symbiosis similar to *S. sjoestedti*. In his opinion, the original symbiont was later lost after the acquisition of the additional yeast. Perhaps this has to do with the fact that the development of the eggs which, in this case, also remain small, follows a completely different course like that in *S. sjoestedti*. Giant cells, which are derived from the inner cellular layer of the secondary covering of the ovarioles, temporarily take up the symbionts which enter the embryo at a much later period. There they are joined by yolk nuclei and thus bring about the development of the first mycetocytes. These giant cells, having lost their function, are reminiscent of the former possession of symbionts with respect to the development of the males. As in the case of *S. sjoestedti*, the noninfected eggs develop into males. However, it appears in this instance that other factors which cannot be comprehended morphologically, determine the infection or noninfection.

Very ancient representatives of the Pseudococcinae, which contain an abundance of forms, are *Macrocerococcus superbus* Leon (9), which lives in the region of the Mediterranean Sea, and *Puto antennatus* Sign., an alpine species that is found on conifers. They take a special position with regard to symbiosis, and are also of considerable interest cytologically. Both of these Coccidae possess a ventral, nonpaired shell-shaped-mycetome that surrounds the ovaries posteriorly. It arises in the primary larvae from polyploid mycetocytes which are occupied by slender, threadlike bacteria. At a rather early period of development the mycetocytes are separated into two categories. The first reproduces rapidly by mitosis. The cells become smaller in the process and the degree of polyploidy is lessened. Subsequently, they enter into intimate union with the ovaries which, at this time, expand. Finally such "secondary mycetocytes" are opened up everywhere between the ovarioles. The remaining "primary mycetocytes," which correspond only to the unitary mycetome of the Pseudococcinae, lose their capacity to divide. Their symbionts degenerate into small pale spheres or they are dissolved.

The "secondary mycetocytes" serve the purpose of transmission. They adhere to the neckpiece of the ovary between the egg and the crown of nourishing cells and, in numbers of eight to nine, penetrate the considerably thickened part of the follicular epithelium into a preformed pit in the anterior pole of the egg (Fig. 9a,b,c, Plate II). In the course of ovarian infection, this type of mycetocyte is completely consumed.

A counterpart thereto is presented by the Aleurodidae. In this family, the migration of mother cells, filled with symbionts, takes place at the posterior pole. However, they degenerate later in the course of embryonic development after the discharge of the symbionts, while in *Macrocerococcus* they remain intact and build the future, primary mycetome. They often reproduce

even after entrance into the egg and at the period of development of the germinal groove they are approached by yolk cells. Shortly thereafter the nuclei of the yolk cells enter the mycetocytes, and even before the young larvae hatch they can be seen lying next to the nuclei of the mycetocytes (Fig. 9d, Plate II). After hatching, both nuclei, which are clearly distinguishable, have fused together. This is the only case of "somatic fertilization" known at the present time, and Buchner is correct in speaking of the "potential immortality" of the mycetocytes. The mycetome of the males of both genera is a rather long oval organ, the inhabitants of which are chiefly small spheres, less seldom the slender, rod-shaped forms. Very often, however, the mycetomes of the males degenerate or are in advanced stages of dissolution.

A completely different type of symbiosis is found in the genus *Rastrococcus* which Ferris in 1950 incorrectly named "*Ceroputo*" and falsely related to *Macrocerococcus* and *Puto* (18). The establishment of a new species (19) is completely justified by the symbiotic findings [Buchner (12, 14)]. *R. spinosus* lives in symbiosis with a delicate, rod-shaped bacterium that is housed in moderately large mononucleated mycetocytes. These bacteriocytes lie scattered about in noncompact groups in the ventral fat body, directly beneath the outer surface of the body. In *R. balinensis* they form compact cellular masses. The infection of the eggs at the upper pole corresponds to that form of symbiosis typical of the Pseudococcinae. After the passage of the bacterial clusters through the raised follicular epithelium of the neckpiece of the ovariole, there accumulates around the fibrous nourishing cord a thick cluster of bacteria which we find later as a spherical mass in the well-defined conception furrow on the upper pole of the egg. However, this condition prevails but a short time. Before the beginning of cleavage the heap of symbionts is flattened out, and this is also typical of the Eriococcinae and Asterolecaniae. Terminal cells of the blastoderm and yolk nuclei then take up the bacteria. These primary mycetocytes are formed into a polar heap of cells before they slide down on the dorsal side of the invaginated germinal strip. During the inversion they are moved ventrally to their final position.

Rastrococcus "Franssenii" possesses, like *Stictococcus silvestri*, yeasts (conidia of an Ascomycete) which surround the considerably enlarged nuclei of the fat cells.

The infection of *Rastrococcus iceryoides* with such yeasts is not so extensive. They occupy somewhat enlarged cells with polymorphic nuclei that are scattered about in the fat tissue. These yeasts are accompanied by small rods which, completely unrestrained, overflow the regions of the fat body devoid of nuclei and, together with the yeasts, infect the eggs.

A fourth type of symbiosis in the Rastrococcinae is represented by *R. "pseudospinosus"*. Here, again, yeasts and bacteria are found as symbionts. The former are housed in giant cells with one or two nuclei which develop from originally but mildly infected fat cells and which are scattered about

loosely in the sterile fat tissue. In this case the bacteria play a minor role and are less easy to observe, except at the time of egg infection, where they can be seen together with the yeasts.

Hippeococcus is also of special interest to the research worker in symbiosis. It is a Pseudococcina which occurs in three species in Java (13).

Contrary to all expectations, Buchner was not able to find an endosymbiosis in these guests of ants, which are found on all possible types of host plants (11). However, the developmental history of these insects shows that in both sexes a spacious mycetome structure is erected which, nevertheless, always remains sterile (Fig. 10, Plate II). The similarity of this sterile "false mycetome," which arises independently of the germinal strip, to numerous mycetomes of the Homopterae is so striking that Buchner does not doubt but that in this case an earlier existing symbiosis was secondarily eliminated. The reasons for this are not known. It may be that the establishment of a different type of nourishment of the lice in the ants' nests was the cause. Of one thing we are sure, namely, that the *Hippeococcinae* first become sexually mature in the ants' nests, and not on the host plants. It is possible that the sap of the sifting tubules alone does not suffice for this stage of development.

This case of a loss of symbionts is not confined to the scales alone. Buchner was able to find symbionts neither in the *Apiomorphinae* (13), which cause the formation of giant blisters on species of *Eucalyptus*, nor in the genus *Matsucoccus*, not in *Coccus cacti*, and he also found no signs of a lost symbiosis.

A short time ago, Buchner, in 1958, discovered a new form of endosymbiosis in representatives of the Hormaphidinae (14, 15). The insect in question is *Cerataphis freycinetiae* v. J. Goot, which he brought back from his trip to Indonesia. This plant louse lacks the nonpaired mycetome so characteristic of aphids, which contains the well-known small, spherical symbionts. Instead, the fat tissue and lymph are penetrated by thin delicate yeasts which form three-membered chains by means of terminal budding. They differ from species to species and are quite large in *Glyphinaphis bambusae*. Perhaps the yeasts enter the fat cells without overloading them or changing them in any way. The means of transmission of these rather large yeasts is the same as for the round, primary symbionts of the Aphidae which make their way over the posterior pole of the egg. Presumably, here, too, the acquisition of yeasts leads to the displacement of the original symbionts. Now and then one can find in the lymph of *C. freycinetiae* slender, threadlike companion symbionts which, however, are never seen in the cells themselves. Further details of this most interesting symbiosis in the Hormaphidinae were worked out by my student Gertrud Kolb; at that time Buchner gave us all of his material for this purpose.

Kolb was the first to study the cytology of the symbionts in detail (31, 32). Although today there is an abundance of literature on the nuclear equivalents of bacteria, accounts of their presence in symbionts were, until 1957-59, rather sparse [Huber-Schneider (23); Frank (17, 21); Rizki (39)].

Resühr, in 1938, and my student, Kotter (33), sought in vain for such structures in the cricket and the body louse, and Bewig & Schwartz were not able to find them in *Haematopinus suis* (3). The chief obstacle in the analysis of the nuclear equivalents of symbionts is the great difficulty in culturing them artificially. There usually remains no other choice than to study them in smears from symbiotic organs or in histological sections. In bacteria that can be artificially cultured, the nuclear structures can best be observed in the logarithmic phase, and this is practically inaccessible in the symbiotic cycle. Nevertheless, Kolb, with an abundance of test material, was able to study the cyclic changes of nuclear substance in the course of the change of form of the threadlike bacteria of the ant, *Camponotus ligniperda*. The DNA-filled structures, demonstrated with the method devised by Piekarski and Robinow and also with the phase-contrast microscope, are present as granules, lying in rows like strings of pearls, in the symbionts of older larvae. The microorganisms themselves are 4 to 11 μ long and approximately 0.5 μ wide. The separate nuclear elements are frequently widened out into cross bars that are often interlaced in the form of dumbbells. In the young puppa, deeply staining rods 2.5 to 5 μ long predominate, and they rise by transverse fission. Such numerous stages of reproduction and the considerable increase in the number of symbionts point to a strong reproductive impulse in this phase of development. At this time, broad symbiotic forms can very often be found in which the chromatic granules are sometimes arranged in pairs in two rows (Fig. 11, Plate III). Frequently, the pairs of granules alternate with the bars, some of which are oriented vertically, others diagonally to the longitudinal axis. In young adults, tennis-racket or spoon-shaped forms are found, such as are characteristic of *Clostridium tetani*. With these there can also be found symbionts with parallel rows of granules. These become more sparse first in four-week-old adults, and one often has the impression that their chromatic substance is arranged in a loose spiral. At this time there appear, together with normal symbionts, strange polymorphic and heavily vacuolated structures, 3 to 4 μ in diameter, that later are apparently changed back into the threadlike forms. Kolb was able to demonstrate for the first time, in 34-week-old adults, typical degeneration and dissolution forms of the symbionts.

Contrary to these positive results with ant symbionts, all attempts to demonstrate nuclear structures in the symbionts of crickets (*Aphrophora silicis*, *Philaenus spumarius*) were in vain, confirming the negative results obtained by Resühr with the symbionts of these insects. The same was found with the symbionts of the Pediculidae. However, in the symbionts of the fruit-fly, *Ceratitis capitata*, Kolb was able to detect DNA which could be completely decomposed with deoxyribonuclease. Small, chromatic granules, only some of which contained DNA, could also be demonstrated in the tubular-shaped symbionts of *Oryzaephilus surinamensis*. Negative results with the Feulgen reaction or with the procedure of Piekarski and Robinow is, according to our experience, no proof that the symbionts are not of a bacterial

nature. For this reason, I cannot agree with Nicol [(37), p. 725] who doubts the bacterial nature of the luminous bacteria because of a negative Feulgen reaction with the components of the illuminating cells of *Pyrosoma*. The *Pyrosoma* symbiosis is one of those cases in which a complete explanation of the symbiotic cycle was possible and this alone could suffice as proof of an illumination symbiosis.

We have Steinhäus and his colleagues to thank for the first investigations of symbiotic microorganisms with the electron microscope (42, 43). He was able to demonstrate the long flagella of the short rods isolated from the cryptic-gut of *Chelinidea vittiger* (Heteroptera).

One year later, Meyer & Frank (36) studied the mycetocytes and bacteria of *Blatta orientalis* with the electron microscope. The cell body, rich in osmophilic substances, is surrounded by a double membrane. In longitudinal sections of the bacteria were found small, osmophilic granules as well as very fine, tubular structures in the partly homogenous, partly nonhomogenous plasma. These granules, found exclusively in dividing cells, are probably identical with the nucleoids, which Frank (22), with the light microscope, found in the symbionts after treating them with ribonuclease. Genuine nuclear equivalents were not present. Very fine pores were observed in the cell membrane. Beside the bacteria, typical mitochondria could be demonstrated which were observed much earlier by Koch (in 1930) and by Brooks & Richards [(4, 38) 1954] with the usual histological methods. Observed for the first time, however, were very small, rod-shaped bodies of unknown function which are present not only in the bacteriocytes, but also in nerve and fat cells.

The taking up of large masses of symbionts into the cell body results in a considerable metabolic burden to the mycetocytes. Coupled with a simultaneous divisional inhibition, this often leads to gigantic growth of the bacteriocytes. This growth is accompanied by an enlargement or an increase of nuclei through mitosis or amitosis. Polyploid nuclei of the mycetocytes are the rule and the polyploidy arises either through endomytotic processes or through subsequent fusion of the daughter nuclei [Baudisch (1, 2)]. The bacteriocytes of *Periplaneta americana* are usually octoploid, but Baudisch found occasional giant mycetocytes with a high degree of polyploidy in *Periplaneta*. The large central nucleus of the compartmental mycetome of *Oryzaephilus surinamensis* L. is 128-ploid, the compartmental nuclei are 64-ploid, and only the small nuclei of the syncytia are diploid. Baudisch also studied the number of chromosomes in the body louse and observed 16-ploidy not only in the embryonic and larval mycetocytes, but also in the nuclei of the fat body and the mid-gut wall, among others.

In the frame of these investigations on the body louse, Baudisch was able to correct and expand our previous knowledge of the embryology and symbiosis in the Pediculidae. For example, the cleavage of the yolk which has long been known but until now had not been described in the body louse, plays an important role in the genesis of the embryonal mycetome. Immedi-

ately after their entrance into the egg, the symbionts gather together at the lower pole. At the beginning of formation of the blastoderm, they are caught by out-runners of the blastodermal cells which lie in this region, are surrounded by them in basket form, and are drawn up closely to the cleavage membrane of the yolk (Fig. 12a, Plate III). This "basket" is a passing arrangement that serves the single purpose of transporting the symbionts to the embryonic mycetome. This transportation of the symbionts is understandable only in the light of the yolk cleavage and the invagination of the germinal groove. With the increasing contraction of the cleavage membrane of the yolk after the tying off of the yolk bundles, the "basket" is drawn more and more into the inside of the egg (Fig. 12b, 13a, Plate III). It finally arrives in the region of the future embryonic mycetocytes that collected early at the upper pole of the egg and there forms 12 to 14 groups of 4 nuclei each. These then enter the last bundles of yolk to be tied off and form a rosette upon which the basket is hung (Fig. 13b, Plate III). The basket opens and releases its contents into the ready and waiting mycetocytes.

Only after the publication of Baudisch's work did we learn of the investigations made by Sander (40), the results of which appeared in a rather inaccessible publication. They deal with the early embryology of the cricket, *Pyrilla perpusilla* Walker. Here the embryonic fate of the symbionts, in the most minute details and including the processes involved in yolk cleavage, is the same as was described above [compare Figures 29 to 34 by Sander (40) with Figures 8 to 11 by Baudisch (2)]. Comparable procedures in insect eggs, in which the symbionts always lie at the posterior pole and where plasma nets and plasma rays play a role in the transportation of symbionts, were described earlier in different insects [Baudisch (2) p. 473; Sander (40) p. 18].

Even before the inversion of the germinal strip in *Pediculus* is accomplished, the future capsule of the larval mycetome is laid down as a mesodermal cell plate between hind-gut and germinal strip. During the rolling back of the embryo, the mycetome arrives in the lumen of the mid-gut. The capsule, still lying under the hind-gut, now migrates forward during a 12 hr. period until it has reached its final position between body wall and the middle portion of the mid-gut. In the meantime, the embryonic mycetome has wandered in the gut lumen to exactly that position where the capsule lies pressed against the gut wall. By means of rhythmic, swinging movements of the capsule which arches forward bowl-shaped at the borders against the gut wall, the embryonic mycetome, together with the part of the gut wall lying beneath, is tied off like a sack. The remains of the degenerating embryonic mycetomes are expelled from the now completed larval mycetome through the narrow slit which allows communication with the gut lumen.

Experiments using the centrifuge, which Baudisch performed on the embryos of lice, showed that a sufficiently long dorsal shifting of the mycetome capsule can prevent the formation of the larval mycetome. Because the symbionts, held back in the gut lumen, are digested after the swallowing of amniotic fluid, lice free of symbionts can be obtained in this way. Thus,

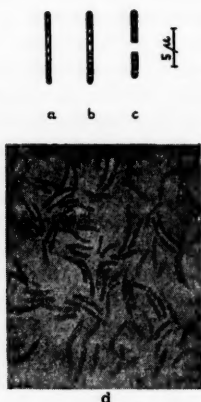


Fig. 11

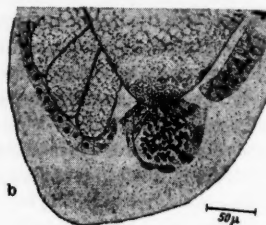
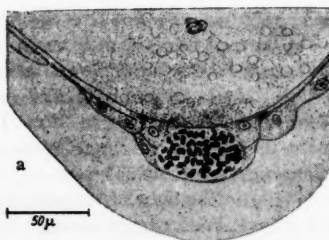


Fig. 12



Fig. 13

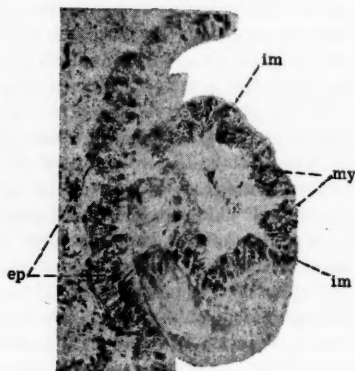


Fig. 14

PLATE III

FIG. 11. *Camponotus ligniperda* Latr. Symbionts from the mid-gut mycetocytes, with rows of chromatic granules arranged like strings of pearls: a: of an older larva; b, c: of pronymphs with cross bars; d: of young adults with parallel rows of granules (after Kolb).

FIG. 12. The formation of the embryonic mycetome of *Pediculus vestimenti* I. a: The symbionts at the lower pole are housed in "baskets." b: the "baskets" migrate at the beginning of invagination into the interior of the egg (after Baudisch).

Aschner's results were confirmed but they received a new interpretation.

In recent years I have published many reviews of the physiological significance of symbiotic microorganisms and the deeper meaning of symbiosis, and a reference to the same should suffice here (25 to 30). These I would like to supplement with a mention of two recent works. In 1959, Jurzitza reported the results of his cultivation experiments with the symbionts of Cerambycidae (24). The yeasts, isolated from the blind sacks of larvae and the intersegmental tubes of female adults (all the yeasts belong to the genus *Candida*), proved themselves to be heterotrophic in regard to vitamins. For their growth the symbionts of *Leptura rubra* L. require thiamine and biotin, those of *Rhagium mordax* Deg., only biotin. Growth of the yeasts of *Rhagium inquisitor* L. is greatly enhanced by the addition of biotin to the synthetic culture medium. In the symbionts of *L. rubra* and *R. mordax*, the formation of dry substance is proportional to the amount of biotin at disposal. In the presence of pyrimidine, the yeasts of *L. rubra* are able to synthesize thiamine. All these symbiotic yeasts give off B-vitamins and amino acids into the substrate. In all probability they supply their animal hosts with these same substances and thus compensate for their lack in the diet of wood. Without doubt, the waste products of metabolism (uric acid, urea, ammonia) are used as nitrogen sources, whereas nitrates and elementary nitrogen cannot be assimilated. It is interesting to note that the characteristic form of the symbiotic yeasts is produced in pure cultures containing urea.

The positive results of the symbiont exchange experiments of Pant and Fraenkel stimulated the pursuit of investigations that Foeckler (20) performed in our institute and which had as a goal the synthesis of a new symbiosis. Test objects were sterile larvae of *Sitodrepa (Stegobium) panicea*, that were raised on a compensating substrate (flour plus 10 per cent yeast extract). These larvae were offered the following yeasts (culture yeasts, wild yeasts, symbiotic yeasts) in the hope of obtaining an infection:

ASCOSPOROGENOUS YEASTS:

Endomyces magnussi
Hansenula californica

ANASCOSPOROGENOUS YEASTS:

Candida mesenterica
Candida reukaufii

FIG. 13. The formation of the embryonic mycetome of *Pediculus vestimentis* II. a: cleavage of the yolk at the lower pole. b: The symbionts shortly before moving in the embryonic mycetome (after Baudisch).

FIG. 14. *Sitodrepa panicea* L. larva. Mycetocytes (my) and intermediary cells (im) of the blind sack, and the epithelial cells (ep) of the mid-gut infected with *Torulopsis utilis* (compare with normal infection, d, in Figure 1, Plate I) (photomicrograph from Foeckler).

ASCOSPOROGENOUS YEASTS:

Saccharomyces cerevisiae
Saccharomyces cerevisiae var. *elipsoides*
Saccharomyces postorianum
Sporobolomyces sp.
Zygosaccharomyces major

ANASCOSPOROGENOUS YEASTS

Cryptococcus albidus
Torulopsis albidia
Torulopsis famata
Torulopsis utilis

SYMBIONTS FROM:

Rhagium bifasciatum
Rhagium inquisitor
Spondylis buprestoides

These yeasts were grown in pure culture in suitable media (ordinary beer wort not mixed with hops; nutrient broth (Difco) with 2 per cent yeast extract) and, in the fresh condition, were smeared on the sterile eggs with a fine brush in the first series of experiments; in the second series they were mixed with the diet (flour) in the ratio of ten parts to ninety parts. However, none of these attempts brought positive results.

Only in one series of experiments, in which the diet consisted of 90 per cent flour and 10 per cent commercial fodder yeast (*Torulopsis utilis* dried at 110°C. on a roller), were the blind sacks of the mid-gut infected with *T. utilis*, but also practically all cells of the mid-gut itself (Fig. 14, Plate III). However, the cells of the blind sacks were not as heavily infiltrated as is the case in normal larvae infected with their own symbionts. Thus, it is understandable that these cells also remain smaller than those of the normally infected mycetocytes. These cells do not lose their cilia, which in the normal case, entirely disappear because of heavy loading of the cells with symbionts.

However, this symbiosis is not a lasting one. In the normally infected larva the symbionts are taken over during the period of puppal rest by the newly formed, much more slender cells of the adult blindsacks, and in the female they are also taken up into the intersegmental tubes and vaginal pockets which serve as the mode of transmission of the yeasts to the offspring. The foreign yeasts, on the contrary, are expelled with the degenerating larval epithelium, and the adults of both sexes are again free of symbionts. Therefore, the artificially induced symbiosis finds its end with the metamorphosis.

An interesting factor in Foeckler's experiments is that the artificial symbiosis, during the larval stage, suffices for storing up the vitamin depots in the fat body to such an extent that all larvae, which hatch from eggs of the former, are able to develop to adults in flour alone (without supplement of yeast extract), and to lay eggs. The eggs, then, of those insects which, in the larval stage were infected with *T. utilis*, received sufficient vitamin reserve to sustain them for an additional life cycle. The F_1 adults are also fertile and lay eggs but the larvae therefrom (F_2 generation) die within two weeks of hatching when kept in flour alone without yeast extract.

The experiments of Foeckler have introduced new problems which, in my opinion, are of considerable interest to the science of immunology. How is it

possible that in the one case the host remains sovereign over its guests while in the other case it appears to lose control over them? In normally infected larvae, a barrier is set up at the border of the epithelium of the mid-gut and that of the blind sacks that is never crossed by the symbionts. How does it come about, that this barrier falls before the foreign yeasts which are taken up in the body? Why do the yeasts now penetrate all epithelial cells of the mid-gut itself, as in the case of parasitism? We do not know the reason. However, we hope that in the not too distant future our efforts to explain this phenomenon will be crowned with success.

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³ Only those works are listed here which do not appear in Paul Buchner's 1953 book on symbiosis (5).

NUTRITION, METABOLISM, AND PATHOGENICITY OF MYCOPLASMAS¹

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A disease was described more than 250 years ago that resembles contagious bovine pleuropneumonia in the pathologic changes induced in the lung and pleura (39). The causative agent was first designated as "the microbe of pleuropneumonia." As other species were isolated they were designated as "pleuropneumonia-like organisms" (PPLO). The group contained many "species" referred to only by origin of isolation. The cumbersome designation "PPLO" was supplanted in the latest issue of *Bergey's Manual* (32) by the generic name *Mycoplasma*.

For many years mycoplasmas have been known to cause disease in animals, yet few scientists have studied them comprehensively. Most research in the field before 1940 was confined to the disease in cattle and goats; little was known about the causative organisms. Since the eradication of contagious bovine pleuropneumonia in 1892, the causative organism from cattle has not been available in this country. The goat strain has not been isolated on this continent.

Following Klieneberger's report (46) on the apparently symbiotic relationship between mycoplasmas (*L*₁) and pleomorphic rods in *Streptobacillus moniliformis* cultures, many investigators began studying mycoplasmas. (The *L*₁ microorganism was later shown to be a variant of the bacillus and not a true *Mycoplasma* (21).) Sabin's (91) demonstration that mycoplasmas caused neurolytic and arthritic changes in mice aroused the interest of pathologists and microbiologists. Following Dienes' (20) finding that mycoplasmas were present in the lower urogenital tract of women, gynecologists began studies on the relationship of mycoplasmas to diseases of the urogenital tract. Another flurry in the study of mycoplasmas followed the demonstration of mycoplasmas in non-gonococcal urethritis (8). Influx of workers into the field was greatest following Markham & Wong's (58) report that chronic respiratory disease of chickens and turkeys was caused by a *Mycoplasma*. The recent finding that mycoplasmas are common contaminants of tissue cultures (89, 90) will force virologists to become acquainted with these organisms.

¹ The survey of the literature pertaining to this review was concluded in January, 1960.

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There have been several general reviews of mycoplasmas in the last twenty years. Sabin (93) thoroughly reviewed the literature in 1941; Edward (25) and Klieneberger-Nobel (47), in 1954, covered subsequent progress. Recently, Freundt (32) described and classified the group in *Bergey's Manual*; in 1958 he thoroughly reviewed (33) the morphology, biology, and taxonomy of the Mycoplasmataceae. Morton (66) wrote a chapter on the mycoplasmas in *Bacterial and Mycotic Infections of Man*. Much information was brought together in a Symposium of the New York Academy of Sciences (124). This review covers only selected topics not discussed in detail elsewhere.

NUTRITION AND METABOLISM

Essentially only three characteristics are considered common to mycoplasmas grown *in vitro*:⁴ colonial appearance, a steroid requirement for growth, and lack of a cell wall. Paradoxically, the absence of a cell wall has not been directly proved and the steroid requirement has not been shown in all strains, e.g., saprophytic strains.

Because Freundt thoroughly covered the subject in 1958 (33), we shall not discuss the properties of mycoplasmas that are used for taxonomic purposes: fermentation of sugars, reduction of methylene blue, liquefaction of gelatin, etc. The point of departure of our survey is the comprehensive and critical review of Edward (25), though certain papers antedating Edward's review are included for better understanding of later work.

Early investigations on the nutrition of mycoplasmas concentrated on concocting media that would support more luxuriant growth. Studies substituting one crude material for another have lingered to the present [Fabricant (28)]. Though such work is of practical importance, it scarcely advances understanding of the nutritional requirements of mycoplasmas, and therefore is not reviewed here.

The basal media employed to grow mycoplasmas usually contain beef-heart infusion, peptone, and NaCl, adjusted to pH 7.8. Such a medium is commercially available (PPLO broth, Difco). To support growth, ascitic fluid or serum is added to the basal medium to a final concentration of 5 to 30 per cent. Sera from different animals vary in their ability to support growth of various strains [Freundt (33)]; some sera are inhibitory or inadequate. Incubation is usually aerobic at 37°C.—a moist atmosphere being provided for plates. Some strains require, or grow better, in an atmosphere containing 10 per cent CO₂ (28).

For a solid medium, agar is added at the level of 1.5 per cent. Liebermeister (53) and Lynn & Morton (57) showed that certain lots of agar were unsatisfactory for some strains of *Mycoplasma*, but the inhibitory component was not identified. For primary isolation, various added agents render the medium selective; most commonly used are the inhibitors penicillin and thallium acetate.

⁴ Strains of *Mycoplasma* were encountered by Yamamoto (126) that could be propagated only in egg embryo.

Many studies on the nutrition of mycoplasmas have dealt with replacement or fractionation of the serum component. Since the fractionation of natural crudes has been fruitless in studies of animal tissue cultures [Morgan (65)], replacement will undoubtedly be the more promising avenue of research.

Edward & Fitzgerald (26) and Edward (24) were able to replace serum with egg yolk and then with an ether extract of egg yolk. The extract, in turn, was replaceable by cholesterol in the presence of bovine albumin or starch. Growth was further improved when the basal medium plus cholesterol was supplemented with an acetone-insoluble lipid (AIL) fraction of yolk. The balance of cholesterol and AIL for optimum growth differed with the various strains used. High concentrations of either component tended to be inhibitory—a situation familiar with other lipid-requiring protists. Cholesterol or stigmasterol could replace cholesterol. Since cholesterol content was not assayed in the test medium after growth, it was not proved that cholesterol was actually taken up by the cells. If it was not taken up, it is conceivable that cholesterol acted by neutralizing a toxic material in the medium. Since both beef-heart infusion and peptones contain sterols, the studies of Edward & Fitzgerald (26) cannot yet be interpreted as rigorous proof of a steroid requirement. Proof of requirement would be growth of the organism, in a defined medium, in presence of a sterol; no growth in its absence. The analogy, however, with other protists shown to require steroids [e.g., van Wagtenonk (121)] makes it likely that the primary requirement is a steroid, with the protein acting either as a stabilizer of the cholesterol dispersion or as the source of additional, toxic fatty acids. Edward & Fitzgerald (26) were the first to demonstrate a cholesterol requirement of mycoplasmas. Since they are the only bacteria to have such a need, mycoplasmas are nutritionally distinct from other bacteria and L-forms. An exception to this criterion may be the saprophytic strains of *Mycoplasma*; their nutritional requirements are currently being studied in our laboratory.

The nutritional requirements of mycoplasmas of human origin have been studied by Smith, Morton, and co-workers. The identity of organisms of human origin is questionable. Recently, Peoples (78), Smith *et al.* (108), and Smith & Rothblat (109) indicated a genetic relationship among three of these strains and certain diphtheroids. Smith *et al.* (108) suggested that the three strains were L-forms that could revert to bacteria. They did not state whether their other human strains were considered L-forms.

In 1951, Smith & Morton (106) reported the isolation by ammonium sulfate precipitation of a growth factor that could replace serum. The factor was assumed to be a low-molecular-weight protein or a high-molecular-weight peptide, since it was both undialyzable and stable at 100°C. for 20 min. at pH 7.0. Several qualitative tests for cholesterol were negative, but their sensitivities were not disclosed. The growth factor was also found in yeast, bacteria, and hemoglobin. The protein was further purified by fractionation on alumina columns [Smith & Morton (107)]. An active fraction (B) had two electrophoretically distinct components, one of which (B-II)

was required by the four test strains. Acid hydrolysis followed by paper chromatography yielded tryptophan, leucine, valine, alanine, arginine, glutamic acid, glycine, lysine, and an unidentified spot. No attempt was made to replace the "peptide" with high levels of individual amino acids to determine whether they could satisfy a peptide requirement (79).

Edward (24) could not grow a strain of *Mycoplasma mycoides* with a serum fraction prepared by the method devised by Smith & Morton (106), but succeeded with a Bacto-PPLO serum fraction (Difco). An ether extract of this material gave a strong Liebermann-Burchardt reaction. The original serum fraction was estimated to contain 150 mg. per cent cholesterol.

Smith *et al.* (104), in a further study of this growth factor, found that it contained protein-bound cholesterol and phospholipid. Their isolation procedure was modified to include a precipitation by heat from a diluted serum solution at pH 6. The lipoprotein was similar to α -1-lipoprotein in isoelectric point and sedimentation constant. The material was replaceable by a mixture of lipid-free protein, cholesteryl laurate, and lecithin. The non-specific requirement for protein could be satisfied by bovine serum albumin or lactoglobulin at ten times the level of lipoprotein. These proteins were found to have high binding capacity for cholesterol.

A critical study by Rodwell (84) on the nutrition of *M. mycoides*, the agent of bovine pleuropneumonia, revealed a requirement for unsaturated fatty acids. In a basal medium containing bovine serum fraction, albumin, acid-hydrolyzed casein, and tryptic digest of casein, serum was replaceable by a combination of the heat-stable protein fraction from serum, cholesterol, and oleate. Osmotic pressure was adjusted to 12 atm. with NaCl, KCl, or sucrose. In such a medium, oleate could be replaced with linoleate or linolenate, and the serum-protein fraction was replaceable by bovine serum albumin or starch. Oleate at a low concentration ($0.5 \mu\text{M}$) or other surface-active substances (sodium lauryl sulfate, deoxycholate, Tween 80, or saponin) lysed the cells of *M. mycoides*; the lysis could be counteracted by either the protein fraction or cholesterol, or both. It would be interesting to find out whether *cis*-vaccinic or lactobacillic acids could replace oleate.

Rodwell (84) showed that the phospholipid in his protein fraction was not essential for growth; he did not report whether the phospholipid was stimulatory for growth. Smith *et al.* (104) reported that phospholipid in the presence of both cholesterol and protein stimulated growth. Essentiality of a phospholipid for growth depends on the basal media used and on the different strains—pathogenic versus non-pathogenic.

Smith & Lynn (105) continued their studies using a methanol-extracted medium. Such a medium gave less basal growth than the unextracted medium, even with supplementary cholesterol and lecithin. Addition of acetate and butyrate brought the growth up to that of the control. The activity of unsaturated acids in this case was not reported. It was found that acetate, squalene, or mevalonic acid, each in one or two concentrations, could not replace cholesterol. Cholesterol or β -sitosterol permitted better growth than did cholesterol, whereas ergosterol, cholesteryl laurate, or epicholesterol

supported poorer growth than did cholesterol. The growth stimulation with ergosterol evidently explains the early findings that yeast cells had growth factor activity. The presence of a growth factor in bacterial cells is less understandable since sterols have not generally been reported to be found in bacteria [Cook (14)]. A few exceptions include *Escherichia coli* and *Azotobacter chroococcum* (19); the quantities of sterol-like material reported, however, were apparently too small (5 mg./100 gm. dry weight of cells) to fulfill the requirements of mycoplasmas.

In considering their results, Smith & Lynn (105) made some generalizations about the steric configuration specificity of the cyclopentenophenanthrene system required by their strains. It appears to us, however, that their conclusions may be premature in light of the following: (a) their crude basal medium, though extracted with methanol, might still contain residual lipids or fatty acids; (b) the additives were tested at only two concentrations.

Smith (103) recently reviewed work on the nutrition of mycoplasmas of human origin. He showed (102) a quantitative relationship between cholesterol esterase activity and the requirement of mycoplasmas for a lipoprotein. Greater activity was exhibited by the strains requiring the growth factor.

Rodwell (85) described a partly defined medium for *M. mycoides* that revealed a requirement for glycerol and lactate in addition to adenine, guanine, thymine, uracil, thiamine, nicotinic acid, riboflavin, and lipoic acid. Growth of another strain was stimulated by pantothenate and biotin in addition to the other requirements.

The requirement for a sterol is absolute but not specific for cholesterol. One effect of cholesterol is to neutralize the toxicity of oleic acid [Rodwell (84)]. This has also been demonstrated with other protists [Hutner (41); Kodicek & Worden (52)]. That a sterol may be an essential metabolite was indicated by growth-response curves with cholesterol in a medium containing oleate [Rodwell (84); Smith & Lynn (105)]. These results are similar to those with *Labyrinthula* (122). Supporting evidence for the cholesterol requirement would be the confirmation of work by Smith & Lynn (105) on the specificity of the steroid requirement by *Mycoplasma*.

Viewed broadly, apparent contradictions in the literature on the nutrition of *Mycoplasma* are paralleled by findings with other protists having lipid requirements. Almost the entire gamut of difficulties encountered in nutritional studies with lipids are epitomized in a paper by Shorb & Lund (97) on Trichomonads. *Trichomonas gallinae* and *T. gallinarum* were found to require cholesterol, two factors from gamma globulin or bovine albumin, another from trypticase, and both saturate and unsaturated fatty acids. Type and concentration of fatty acids were the most critical requirements. The organisms were stimulated by a fourth factor from serum. The work bears out an assertion that problems of lipid nutrition are ubiquitous. Shorb & Lund (97) elucidated the interactions of essential saturated and unsaturated fatty acids in the nutrition of their organism—a problem that undoubtedly figures in the nutrition of mycoplasmas.

Further studies on the lipid nutrition of mycoplasmas await the availability of a chemically defined basal medium, more basic knowledge of the factors making for the accessibility of lipid nutrients, and use of antioxidants with fatty acid supplements. The lack of such synthetic media has precluded attempts to define the *in vitro* conditions for best preservation of surface antigenicity, immunologic studies, and virulence.

Osmotic pressure.—Some students of mycoplasmas contend that these organisms are similar to L-forms [Smith & Rothblat (109); Dienes & Weinberger (22)]. It would thus be of interest to find out whether mycoplasmas, like L-forms, require media of high osmotic pressure. Rodwell (84) demonstrated that *M. mycoides* grew in only a limited range of osmotic pressures, with the optimum at 12 atm. We have made a similar finding with avian mycoplasmas, the optimum for one strain being at about 10 atm. (unpublished data). Rate and total growth decreased with increased osmolality of the medium. These pressures are much lower than those required to maintain viable L-forms.

The absence of a cell wall in mycoplasmas has been assumed, though direct evidence is lacking. The assumption is incompatible with the presence of "mycelium" in certain species. Possibly the "envelopes" of filamentous and coccoid mycoplasmas are chemically different. Recently, Plackett (82) reported on the absence of significant amounts of hexosamine and diaminopimelic acid in hydrolyzed cells of *M. mycoides*. He concluded that a "muco-complex" was lacking in *M. mycoides*. Firm information on the relationship between *Mycoplasma* and L-forms requires study of ultrathin sections and of the chemistry of the envelopes of both forms. Cytologic studies are needed to show the mode of multiplication of mycoplasmas, and whether they have cell walls. Knowledge of the constituents of the envelope would enable comparison with membranes of bacteria and might elucidate any relationships between mycoplasmas and bacteria.

That the osmotic sensitivity of *Mycoplasma* is due to "lack" of a cell wall is mentioned in many papers [e.g., Freundt (33)]; the assumption is not supported by published data, however. From a study of the effect of osmotic environment on the viability of human strains, Smith & Sasaki (110) concluded that suspension fluids of low osmotic pressure did not significantly decrease viability. Their results were not clear-cut, which makes interpretation difficult.

Gossling (34) recently described a high mortality for bacteria subjected to changing ionic environments. Thus, the indirect approach, proving absence of cell wall in mycoplasmas by showing an increased osmotic susceptibility, seems inconclusive.

A further question: Are mycoplasmas, especially the coccoid forms, related to "stabilized" protoplasts? Unveiling any relations between mycoplasmas and protoplasts awaits the time when we know more about the nutrition and metabolism of both groups.

Metabolism.—Dissimilation of carbohydrates by fermenting strains of *Mycoplasma* was studied independently in three laboratories. Detailed

studies on the pathway of monosaccharide breakdown were made only with *M. mycoides* [Rodwell & Rodwell (86, 87, 88)]. Aerobically grown cell suspensions of *M. mycoides*, in the presence of glucose, formed CO₂, pyruvate, and acetate. Pyruvate, lactate, and glycerol were oxidized to CO₂ and acetate. The oxidation of glycerol was enhanced in the presence of catalase. Anaerobically, glucose was not attacked; pyruvate was dismutated to lactate, acetate, and CO₂.

The end products of glucose fermentation were determined by Tourtellotte & Jacobs (118) with strains of human, bovine, caprine, ovine, porcine, avian, and sewage origin,⁵ and by Neimark & Pickett (69) with *M. laidlawii*, *pulmonis*, *neurolyticum*, *fermentans*, *gallisepticum*, *gallinarum*, and kid, calf, and avian strains. Tourtellotte & Jacobs (118) observed that cells growing aerobically with glucose formed CO₂, lactate, pyruvate, and acetate; traces of acetyl methyl carbinol were detected. Pyruvate did not accumulate when yeast extract was added to the medium. Neimark & Pickett (69) reported that aerobically growing cells formed only lactate and CO₂. The discrepancy in results is not due to differences in strains, since some strains were studied by both groups. It possibly lies in the use of different supplements in the growth medium, i.e., serum fraction [Tourtellotte & Jacobs (118)] and horse serum [Neimark & Pickett (69)].

Both the homo- and hetero-lactic fermenting strains, except *M. neurolyticum*, attack glucose anaerobically; they thus differ from the type species, *M. mycoides*, which lacks this ability. No data are available on the carbohydrate metabolism of non-fermenting strains of *Mycoplasma*.

Lack of a tricarboxylic acid cycle in *M. mycoides* was suggested by the inability of cells to metabolize intermediate compounds of the cycle, and by the absence of a cytochrome system [Rodwell & Rodwell (86)]. Lynn (56) (with human strains), and Tourtellotte & Jacobs (118) (with strains aforementioned), concluded that the tricarboxylic acid cycle played a minor role, if any, in the metabolism of their strains.

In general, mycoplasmas are aerobic in their growth requirements, yet possess the metabolic makeup of facultative microorganisms. They have exacting nutritional requirements and limited synthetic capabilities, and in these respects are similar to certain species in the genus *Streptococcus*. Serologic studies may elucidate a relation between the mycoplasmas and the streptococci.

Amino acid metabolism.—Amino acid utilization, the appearance of nitrogenous products, and the "glutamine pathway" in non-fermentive human strains were studied with paper chromatography by Smith (98). Experiments on the uptake of amino acids by resting cells showed that arginine, L-glutamic acid, and L-glutamine were utilized almost completely; aspartic acid, histidine, leucine, threonine, tryptophan, and tyrosine were used to varying degrees [Smith (98)]. Rodwell (85), in contrast, observed that only serine and threonine were metabolized by his strains; transaminases

⁵ Taxonomically unnamed strains are referred to by the origin of their isolation.

could not be detected. Cell-free preparations of strain 39 of human origin hydrolytically deamidated glutamine—optimally at pH 8.0; glutamine was deamidated phosphorolytically with concomitant production of ATP—optimally at pH 6.0 to 6.5 (100). Demonstration of this reaction with a purified enzyme would be of great importance as an energy-yielding mechanism not hitherto described for bacteria. Such a system has been described in detail for animal and plant tissues [Meister (60)]. Further, Smith (99) demonstrated glutamic acid conversion (by strain 39) through Δ -pyrroline-5-carboxylic acid to proline; optimum activity was pH 7.5. The occurrence of this pathway in other microorganisms was reviewed by Meister (61). Smith (101) showed (with strains 07 and 39) that citrulline, through a phosphoroclastic cleavage, yielded ornithine and adenosine triphosphate. Carbamylphosphate was an active intermediate in this reaction, which had optimal activity at pH 5.5.

The above work was done with two human strains (07 and 39), chosen because of their ability to grow in a chemically defined basal medium (Tissue culture medium No. 199, Difco) fortified with a lipoprotein growth factor. The results, therefore, cannot yet be applied to any but nutritionally sufficient strains. Recently, Smith (103) reviewed his work on amino acid metabolism of mycoplasmas. As seen from the foregoing, the special adaptation of mycoplasmas that permits their growth in specific tissues of animals remains wholly unknown.

PATHOGENICITY

Little is known about the mode of transmission of mycoplasmas of animal origin. The majority of mycoplasma infections occur by direct contact between infected and susceptible animals. There is limited evidence of transmission through air-borne route or fomites. The pathogenic mycoplasmas of fowl are transmitted through the infected egg [Van Roekel *et al.* (120)]. The rate of transmission from individuals was highest during the acute stage of disease. "Chronic carriers," without clinical signs of the disease, often disseminate mycoplasmas.

Mycoplasmas isolated from the genitourinary tract of men have not been shown to produce disease. Freundt (33) made extensive studies on transmission of these organisms. In humans the major mode of spread was through sexual contact. The organisms could become established in the vagina when the pH was above 4.9. Bacterial infections that raised the pH of the vagina enhanced the growth of mycoplasmas. The male urethra, because of its high pH and availability of nutrients, was a good site for growth of the organism. These organisms were present in individuals in about the same percentage, whether or not urethritis was present.

M. mycoides var. *mycoides* is transmitted naturally by direct contact. Campbell (11) developed a reproducible procedure for the production of pneumonic lesions by exposing cattle to a small-particle aerosol of culture from a paint sprayer.

The pathologic changes induced by *M. mycoides* have been thoroughly

described by Meyer (62). Typical lesions in the lungs are usual in acute cases: a marbled appearance, with pink, dark red, and gray lobules surrounded by thickened interlobular septa. The pathologic changes depend on the stage of the disease. The process can extend to areas of necrosis completely surrounded by a thick wall of connective tissue. Histologically the lobular changes are essentially those of fibrinous pneumonia. According to Meyer, mycoplasmas primarily affect the connective tissue. The organisms invade and multiply in the lymph vessels, with subsequent lymphangitis and lymphothrombosis. The process extends from the lymphatics to adjacent blood vessels, resulting in various forms of vasculitis and thrombi formation. Local necrosis is the result of blockage of the blood supply. Meyer considered the perivascular deposition of fibrin and leucocytes to be characteristic of *M. mycoides* var. *mycoides* infection.

Meyer (62) also described a polyarthritis in cattle produced by intramuscular inoculation of *M. mycoides*. Involvement of the joints appears to have developed by way of the lymphatics or blood vessels. Gross observation revealed modest erosion of the synovial linings and excessive fluid with flakes of fibrin. Microscopically there were areas of leukocytic infiltration in the synovial cavity. There was extension of the process to the connective tissue layer. The vascular changes were capillary distension without the occlusion previously described.

Inoculation of *M. mycoides* by the intraperitoneal route results in severe serofibrinous peritonitis, which seems to follow the same vascular changes seen in the lung lesions. Similar lesions are observed after intramuscular inoculation. For detailed descriptions of histologic changes in the lymph nodes, liver, spleen, and intestinal tract, see the thorough study by Meyer (62).

Sheep, goats, and buffaloes are susceptible to inoculation of the organism [Henning (39)] and mice [Hyslop (42)]. Horses and swine are refractory to inoculation with *M. mycoides* var. *mycoides*. The organism is very lethal to embryonating chicken eggs; it produces edema and plaques on the chorioallantois [Tang *et al.* (116)].

Recently, a *Mycoplasma* other than *M. mycoides* var. *mycoides* was isolated from a calf with arthritis [Moulton *et al.* (68)]. The calf was in poor condition, with markedly swollen joints. Clinical examination disclosed involvement of lungs, pleura, and other tissues. At necropsy the enlarged joints had an excessive amount of yellowish viscid fluid and fibrinous plaques. The lungs had lobular areas of red hepatization. A severe conjunctivitis noted in one calf was apparently incidental to the mycoplasmal infection. Tissue sections of the joints revealed a fibrinous arthritis. The organism was isolated from the joints, kidneys, and spleen.

Lambs that received an intravenous dose of the *Mycoplasma* showed conjunctivitis and no other signs of disease. Mycoplasmas were not recovered from the lambs at necropsy.

Two pigs inoculated intravenously and white mice inoculated intraperitoneally failed to show signs of disease or pathologic changes, clinically

or at necropsy. Embryonating chicken eggs were killed by yolk sac inoculation of the culture.

Mycoplasma agalactiae is spread by direct contact and through infected milk and exudates [Frei (31)]. It produces lesions of the mammary glands, joints, and eyes of goats and sheep. Milk production is reduced. Blood elements are found in the milk. Painful articular involvement may be evident, and several forms of conjunctivitis are seen. The primary changes in the udder are interstitial mastitis, with eventual fibrosis. The organism apparently attacks connective tissue around the joint, but without penetrating to the synovia. The lesions of the eye are hyperemia of the conjunctiva and sclera, with suppurative changes in the anterior chamber. Chicken embryos are killed by *M. agalactiae*. Laboratory animals are not susceptible to this infection.

The second pathogenic *Mycoplasma* found to affect goats, described by Longley (54), is evidently directly transmitted. Because the disease resembles infectious bovine pleuropneumonia, the organism was named *M. mycoides* var. *capri*. In goats this *Mycoplasma* incites pneumonia, synovitis and, under traumatic insult, cellulitis. No detailed histologic studies have been made of this mycoplasmal infection. Cattle and laboratory animals are not susceptible to *M. mycoides* var. *capri*.

Cordy *et al.* (18) described a *Mycoplasma* from goats that was transmitted by direct contact under field conditions during the stress of kidding. Experimental exposure of healthy sheep and goats to moribund animals failed to incite disease [Cordy & Adler (16)]. Spraying the organism into the nares established the infection in sheep. The mycoplasmas produced septicemia and arthritis in goats, sheep, and swine. Culturally the organism resembled *M. mycoides* var. *capri*, but the absence of pneumonia in naturally and laboratory-infected animals argued against this classification. The sign characterizing this disease was lameness from obviously swollen joints. Temperatures in affected animals rose as high as 107.3°F. At necropsy the lesions were consistent with septicemia, and a constant feature was fibrino-purulent arthritis, many times affecting almost all synovial joints. Free fibrin was observed in the peritoneal cavity in experimentally exposed animals and in field cases. Other septicemic lesions were enlarged spleen and lymph nodes, occasional fibrinous pericarditis, and necrotic foci in the liver. Microscopic examination of the joints revealed a fibrino-purulent arthritis. Cordy *et al.* (18) described a severe cellulitis that seemed to follow any trauma. The eye lesions were a mucopurulent conjunctivitis.

The pathogenicity of the organism for the pig was considered to be quite unusual in view of the host specificity of mycoplasmas. Signs and lesions similar to those described for goats and sheep were found in four pigs inoculated via the anterior vena cava and in two pigs exposed intraperitoneally [Cordy *et al.* (17)]. Yamamoto *et al.* (129) propagated this agent in chicken embryos and found it extremely lethal. Plaques produced on the chorioallantois were similar to those described for *M. mycoides* var. *mycoides*.

The organism grew so readily in embryos that 1×10^{11} organisms per milliliter were found in the allantoic fluid in some instances.

The high pathogenicity of this organism for an unrelated host, pigs, makes remarkable the insusceptibility to inoculation of a horse, dogs, calves, turkeys, chickens, rats, mice, guinea pigs, and rabbits.

Boidin *et al.* (9) described mycoplasmas that were originally isolated from lung lesions of sheep. Intratracheal inoculation of cultures in serum broth produced fibrinous arthritis without pneumonia. The hock and stifle joints were usually enlarged, and contained fibrous exudate in one of four sheep exposed. As with the organism described by Cordy *et al.* (18), not all joints were involved. The disease was much more transient and mild.

Mice inoculated by the intravenous, intranasal, intraperitoneal, and intracranial routes exhibited no visible disease. Embryonating chicken eggs were killed by inoculation with this *Mycoplasma*. The major lesions were cutaneous hemorrhages.

Switzer (112, 113) isolated a *Mycoplasma* (*M. hyorhinis*) from the turbinates of swine with atrophic rhinitis. It is uncertain how this organism is transmitted since intranasal inoculation of the agent produced no disease. Switzer did, however, notice a mild conjunctivitis in the pigs that received the nebulized amnio-allantoic fluid. Administration of the organism intraperitoneally into five pigs resulted in severe fibrinous pericarditis and peritonitis. One of the pigs developed a lameness and had excessive fluid in the joint. Switzer (114) later indicated that pigs were most susceptible when less than six weeks old. Further observations of field cases of fibrinous pericarditis revealed the organism in pooled pericardial and pleural exudate in eight of nine accessions. Chicken embryos killed by inoculation of this organism exhibited severe myocarditis and pericarditis. Plaques were found on the chorioallantois. This strain is cytotoxic for swine kidney cells [Switzer (115)]. Mice, guinea pigs, chicks, rabbits, poult, a calf, and a lamb were insusceptible.

At least three mycoplasmas produced disease in mice. All seem transmitted by direct contact. *M. neurolyticum* is the etiologic agent of brain lesions [Sabin (91)]. This *Mycoplasma* has been found in the conjunctiva and nasal mucosa, which would allow ready contact infection [Sabin (92)]. Sabin (91) produced brain necrosis in mice by intraperitoneal or intrathoracic inoculation. He later showed that the neurolytic changes were due to an exotoxin produced by the organism. The major sign of disease in young mice is a rolling over in the long axis of the body. Old mice may develop a synovitis. There appears to be considerable difference in virulence of strains. Findlay *et al.* (30) isolated a strain of *M. neurolyticum* that did not kill mice by inoculation via the intraperitoneal or intrathoracic routes.

Mycoplasma pulmonis is found in bronchiectatic lung lesions in old rats [Klieneberger-Nobel (48); Klieneberger & Steabben (50)]. Artificial production of the bronchial occlusion, followed by inoculation of the mycoplasma, produced disease in rats [Klieneberger-Nobel & Cheng (49)]. There is a

question regarding the pathogenicity of this strain for rats, particularly in view of unsuccessful attempts to produce the disease in normal rats [Klieneberger & Steabben (51)]. In mice, however, the organism is associated with chronic respiratory catarrh, and the disease is readily induced by contact or nasal instillation of the organism [Edward (23)].

M. arthritidis is the cause of arthritis in rats and mice. Intranasal and intracerebral inoculations of this strain produced pneumonia and encephalitis in mice but not in rats. Contact exposure failed to produce disease. Collier (13) described a gross swelling of the tibiotarsal joints in rats. The infection progressed to tense swellings of the joints that could extend to other articulations. The cut joint shows a large amount of purulent exudate. X-ray pictures by Collier show marked enlargement of the affected joints. Subcutaneous inoculation incites massive necrosis in mice and focal abscession in rats. Rabbits and guinea pigs were not affected by exposure to *M. arthritidis*.

Mycoplasmas have been isolated from pigeons with coryza [Mathey *et al.* (59)]. Serologic studies indicated that the strain was distinct from *M. gallisepticum*. This *Mycoplasma* produced coryza in pigeons by intranasal instillation of culture. Chicken embryos were readily killed by yolk sac inoculation of the organism.

Two mycoplasmas produce disease in fowl: *M. gallisepticum* and an unnamed strain. They have been transmitted by contact and through infected embryos. In laying hens, ovule infection can follow invasion of adjacent air sacs, and the organism then becomes egg-borne.

M. gallisepticum was shown to be the cause of a chronic respiratory disease of chickens and turkeys [Markham & Wong (58)]. The disease was characterized by infraorbital sinusitis, rales, conjunctivitis, retarded body growth, and decreased egg production. At necropsy the infraorbital sinuses of turkeys were filled with a gelatinous exudate. The lesions in chickens were only moderate distension of the infraorbital sinus, with purulent exudate. In acute stages of the disease, catarrhal tracheitis was seen. The air sacs were thickened and covered with yellow, thick exudate that sometimes became caseous as the process progressed. The joints had large amounts of purulent exudate. Histopathologically the primary lesions in areas other than the joints were a lymphofollicular reaction, with lymphoid cells dispersed around the foci [Jungherr *et al.* (43, 44)]. Microscopic examination of the joints revealed thickening of the synovial membranes and accumulations of heterophiles in the sac cavity [Adler (2)].

Cordy & Adler (15) produced the encephalitis in poults by intravenous inoculation of culture. Clinically the affected birds were hyperexcitable, and many had torticollis. Near death, the turkeys were somnolent. The microscopic lesions of the brain were focal necrosis from vascular occlusion.

M. gallisepticum was found in diseased partridges, pheasants, and pigeons [Van Roekel *et al.* (119)]. It also has been isolated from pea fowl [Wills (125)]. The primary manifestation is a catarrhal discharge from the nares, with occasional purulent air sacculitis.

From day-old poults a *Mycoplasma* was recovered that did not have the

pathogenic or antigenic characteristics of the other mycoplasmas of fowl [Adler *et al.* (3)]. This strain produced air sacculitis in poults and growing turkeys. Chicken embryos supported growth of the organism but were not killed. Chickens were not susceptible to this *Mycoplasma* by intravenous, intracardial, intraocular, intracerebral, or intraperitoneal inoculation.

Although *M. gallinarum* has been designated the type species for fowl [Freundt (32)], it is not pathogenic to chickens or turkeys [Adler *et al.* (6)]. Its lethality for chicken embryos is at the same level as that of *M. gallisepticum*. Its possible role in mixed infections has not been clarified.

M. iners was not pathogenic to chickens or turkeys by intracerebral, intraperitoneal, intracardial, or joint inoculation [Yamamoto & Adler (128)]. The organism was lethal to embryos, with the production of arthritis. This *Mycoplasma* was a good agent for the study of the pathogenesis of arthritis. Moulton & Adler (67) described the histopathologic changes induced by *M. iners* in chicken embryos: The lesions progressed from hyperemia to necrosis of the synovial layer and articular cartilage. Mononuclear cells accumulated in the synovial sac later in the process.

Adler (1) isolated a *Mycoplasma* from a thickened air sac of a parakeet. An antigen prepared from this strain was agglutinated to a titer of 1:640 by sera of affected parakeets. The sera did not agglutinate antigens prepared from four different mycoplasmas of poultry origin, and the immune sera from these poultry strains failed to agglutinate the antigen prepared from the parakeet strain. Nine serial passages *in ova* failed to enhance virulence to the chicken embryo, and no mortality occurred despite the large numbers of organisms present in the yolk.

From the foregoing descriptions a general pattern of disease production is suggested. Most mycoplasmas enter the host via the respiratory tract. The organisms may affect the respiratory tissues directly or proceed to the joints by way of the lymphatic channels or blood stream. Arthrotropism is a consistent characteristic of many pathogenic mycoplasmas. Extension of infection from the respiratory system to the eyes is also fairly common. Two of the pathologic aspects of Reiter's syndrome in man—arthritis and conjunctivitis—are commonly seen with other mycoplasma infections of animals, yet much of the effort to determine the etiologic role of these organisms has been unsuccessful. Failure to isolate mycoplasmas from joints in Reiter's syndrome or from arthritides is possibly due to rapid disappearance of the organism from the affected joint or the very small number present. If the organisms were found, further verification of their role could be acquired by serologic tests from serum antibodies during the course of infection.

MIXED INFECTIONS AND STRESSING FACTORS

An important aspect in the pathogenicity of many of the mycoplasmas is the enhancing effect of other infectious agents and stressing factors. Nothing is known of the role of exalting infections or stress in mycoplasma infections of man.

Mooser (63, 64) observed a marked enhancement in the growth and

pathogenicity of murine mycoplasmas associated with ectromelia virus. Findlay *et al.* (30) found that the English strain of *M. neurolyticum* disappeared from the liver and spleen three days after intraperitoneal inoculation, but, in association with subcutaneous inoculation of a carcinoma, was maintained in the tissues. Howell *et al.* (40) showed that *M. arthritis* produced arthritis regularly in conjunction with a degenerating lymphosarcoma. A viable tumor did not have this effect. Nelson (73) showed that *M. pulmonis* grew in the brain of Swiss mice if the mouse hepatitis virus was injected simultaneously. Mycoplasmas or virus infection did not produce disease alone.

M. agalactiae is more active in debilitated animals under field conditions; it is most common at lambing or kidding time [Todd (117)]. The goat strain described by Cordy *et al.* did not become a herd problem until onset of the kidding season, though the disease had been seen in one buck several months earlier (18). In every case the does were affected following parturition or after the severe drain of lactation. Greatest losses in kids followed the stress of weaning.

Boidin *et al.* (9) found an agent of the psittacosis-lymphogranuloma group in affected lungs. *Pasteurella multocida* also was isolated from some of these sheep. None of the aforementioned agents, singly or in combination, incited typical disease. Extensive pneumonia was induced only by inoculating ground lung material containing all the agents. Possibly the lung contained an organism not yet identified.

Investigations of outbreaks of chronic respiratory disease (CRD) in chickens and turkeys have usually revealed in the tissues of infected birds a *Mycoplasma* and one of a number of different viruses affecting fowl. Evidence from studies of the infectious bronchitis virus in combination with *M. gallisepticum* indicate that disease in chickens is more severe from the two agents acting in harmony than from either agent alone [Grumbles *et al.* (37)]. Latent egg-borne *M. gallisepticum* was activated by infectious bronchitis virus under natural and experimental conditions. Vaccination with infectious laryngotracheitis sometimes produced severe hemorrhagic tracheitis in birds with mycoplasmal infection of the trachea.

Fahey & Crawley (29) reported that an unidentified virus was a necessary part of the CRD syndrome. According to these authors, neither the *Mycoplasma* nor the virus alone was capable of producing disease; it took both agents acting in concert to induce typical CRD.

Bacteria also have been shown to exalt mycoplasmal infections. Such observation was first made twenty-five years ago by Nelson (70, 71) who investigated the etiology of infectious coryza. He found both *Hemophilus gallinarum* and a *Mycoplasma* in infected birds. The infection caused by *H. gallinarum* was acute and did not persist beyond 15 days. The disease produced by the *Mycoplasma* was more severe and persisted for several months. Adler *et al.* (4), twenty years later, obtained essentially the same results with recent isolates of strains of *M. gallisepticum* and *H. gallinarum*. Gross (36) indicated that *Escherichia coli* infection could cause complicating infection in CRD. The *Mycoplasma* alone caused a mild tracheitis and aro-

sacculitis; *M. gallisepticum* and *E. coli* together incited severe cases of aeroculitis, fibrinous perihepatitis, and pericarditis.

Not only viral and bacterial agents are incriminated as inciting agents of mycoplasmal infections; chemicals have also been shown to increase disease severity. Field observations have shown that subcutaneous application of diethylstilbestrol excites latent *M. gallisepticum* infection into obvious disease; administration along with *Mycoplasma* gives more uniform infection.

Shifrine *et al.* (96) recently demonstrated that a subinfective dose of *M. gallisepticum* produced obvious disease in chickens fed histamine; birds fed normal diets were unaffected.

IMMUNE RESPONSE

Serum from recovered animals seems to protect against experimental infections unless the disease has progressed to visible clinical signs. Then the animal cannot be protected with immune serum. The effect of this type of passive immunity was thoroughly reviewed by Sabin (93). The active immunity induced by vaccination has, in most cases, been shown to be induced with living attenuated cultures, though Bridre & Donatien (10) found that a living culture could not be used either with immune serum or even after attempted attenuation through 100 passages *in vitro*. Later, Zavagli (130) immunized with an aluminum hydroxide-adsorbed formalin-inactivated vaccine containing infected milk, brain, and other tissues. This product had an advantage in countries where commercial vaccine production was difficult; an affected animal could be sacrificed and its tissues used to immunize the animals in each region. Another killed product, used for contagious agalactia, was developed by Lopez & Lopez (55). They found good protection with a formalin-killed chicken embryo-propagated vaccine of a local strain. Shamir (95), in a preliminary report, said that *M. agalactiae* was attenuated by serial passage in embryonated chicken eggs. A vaccine prepared from the fortieth egg passage produced no clinical disease in 27 goats and 2 sheep. Vaccinated goats were immune to challenge with 10 lethal doses of virulent culture 2.5 months after vaccination. Sabin & Morgan (94) found that vaccination with concentrated suspensions of *M. anthropicus* (mouse type B microorganisms) killed by heating 30 min. at 50°C. protected a majority of mice after they had shown clinical signs of disease. Killed mycoplasmas failed to immunize cattle against the prototype disease. Walker (123) introduced the use of attenuated cultures for immunizing cattle. Usually, a twenty-fifth passage of the organism in an enriched medium was satisfactory for immunization but was not virulent enough to produce disease. Both success and failure followed vaccination with cultures attenuated *in vitro*; and reactions to a vaccine seemed to differ from country to country. The reason could be differences in the native resistance of animals in various areas, and that other diseases might interfere with the immune response to vaccination. Priestley (83) recently reported on extensive immunization studies with cattle in Africa. Incidence of the disease decreased appreciably in areas in which cattle were immunized. He prepared a lyophilized product since the

use of wet cultures was impractical. Many of the problems faced under difficult field conditions appear to be due to the instability of these organisms. According to Gray & Turner (35), at least 10^6 organisms were required to immunize cattle. Lyophilization reduced the population as much as 99 per cent. Stabilizers were used to decrease losses from lyophilization, and the amount of fluid was kept down by concentrating the organisms by centrifugation and resuspending in saline or serum before lyophilization. Egg-propagated vaccines have given a significant degree of protection but not complete immunity (81). Piercy (80) recently pointed out the problems of immunization against *M. mycoides* under various field conditions.

ISOLATION AND IDENTIFICATION OF MYCOPLASMA

The isolation of mycoplasmas from infected tissues is difficult because of the exacting requirements of these organisms. The organisms often fail to grow when seeded directly on an agar medium. Usually, "blind" passages in enrichment broth, eggs, or tissue culture are required before growth is possible on agar medium. In some instances, the only means of identifying a particular strain may be by inoculating susceptible animals in order to observe typical gross and microscopic lesions. A serious complicating factor in isolating mycoplasmas is their prior presence in so-called normal animals, tissue cultures, and embryonating eggs. Embryonating chicken eggs are naturally contaminated with egg-borne organisms [Van Roekel *et al.* (120)]. Tissue culture, first used by Nelson (72) for isolating his coccobacilliform organism, is an excellent medium for the growth of mycoplasmas. Several workers [Rothblat (89)] have shown, however, that many tissue cultures from different sources were naturally contaminated with mycoplasmas or L-forms of bacteria. The use of laboratory animals and birds also presents numerous problems, because mice, rats, chickens, turkeys, sheep, cattle, and swine can be carriers of mycoplasmas. Another factor to be considered is the presence of both pathogenic and non-pathogenic organisms in the tissues of test animals. With few exceptions, the non-pathogens are more readily cultured. This was demonstrated by Adler *et al.* (5) in determining the etiology of infectious sinusitis. Two strains of *Mycoplasma* were present in the infraorbital sinus exudate. One strain, readily propagated on artificial media, was non-pathogenic for turkeys and chickens. The second strain, which did not grow in serum-enriched media, was capable of producing disease.

Reproduction of disease from serial culture has many times been difficult. The reason in some instances may be that non-pathogenic organisms recovered from the infected test animals grew to predominance. There is selection for pathogenic organisms in susceptible hosts; however, not all of the non-pathogenic strains were killed in the tissues of the susceptible host. Therefore, it is necessary to use either mycoplasma-free or tested materials and animals.

Hearn *et al.* (38) recently described procedures for detecting mycoplasmas or L-forms in tissue cultures and for eliminating them. The organisms were

detected by direct culture on agar or through enrichment broth and subculture to agar. The contaminants were eliminated by treating initial line cultures with 100 to 200 μ g. per ml. of chlortetracycline and filtering all media through fine-porosity pads. The development of L-forms in the tissue culture or inoculum was later prevented by using no antibiotics.

Often the only means of detecting previous exposure to mycoplasmas was by testing for specific antibodies. Agglutination [Newing (74)], hemagglutination [Jungherr *et al.* (45)], and complement-fixation [Campbell & Turner (12)] tests were used. However, infected animals may not have detectable antibodies.

The variation in sensitivity of the antigens has been a major problem in serologic tests. Some strains of mycoplasmas were poor antigens. It may be necessary to prepare several antigens from the same serologic type and select one for testing the experimental animal. The growth phase of harvest of the antigen is not critical for the agglutination test. It is critical, however, for the hemagglutination test.

When an animal is serologically negative for mycoplasmal antibodies, culturing the organisms from different sites may be necessary to ensure that the animal is not infected. Isolates should be tested serologically to determine their relation to the mycoplasmas used in inoculation.

CHEMOTHERAPEUTICS

Many antibiotics and chemicals have been tested for their efficacy in preventing the growth of mycoplasmas *in vitro*, *in ovo*, and *in vivo*. Of these, the *in vitro* tests, using agar diffusion or broth cultivation methods, are useful for rapid determination of the effectiveness of a drug against mycoplasmas. Zolli *et al.* (131) used fermentation of maltose in a phenol red broth to determine sensitivity to different drugs of a number of mycoplasmas from fowl. This procedure proved of particular value in studying many products with strains that utilize sugars, since growth of the mycoplasmas was revealed more readily by pH change of the phenol red broth than by visible growth.

The *in ovo* method has two major advantages over the *in vitro* procedure: (a) insoluble compounds can be tested after suspension in fresh yolk; (b) the embryonating egg offers a living system for initial evaluation of toxicity as well as for mycoplasmal inhibition studies [Yamamoto & Adler (127)]. Since the activity of a drug indicated by the two preceding procedures is not matched [Olesiuk *et al.* (75, 76)] *in vivo*, final evaluation of a compound must be conducted in the host in which the drug will be used.

Both drug-resistant and susceptible mycoplasmas have been encountered among strains of *M. gallisepticum* [Yamamoto & Adler (127)]. Several mycoplasmas should then be used to test the efficiency of a given drug. A further complication in evaluating drugs against mycoplasmas is that the results obtained for a given species apply only to that species and not to other species, e.g., erythromycin prevented growth of mycoplasmas of avian, goat, and sheep origin but was ineffective against mycoplasmas of human origin [Stokes (111)]; viridogrisen was active against rodent [Ehrlich *et al.* (27)]

and avian mycoplasmas but inactive against one isolated from goats [Yamamoto *et al.* (127); Adler *et al.* (7)].

Drugs against mycoplasmas have been found to be bacteriostatic; none were found to be bactericidal. Recently, it was shown that antibiotic-resistant strains resulted from treatment [Osborn *et al.* (77)].

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INTERPRETATION OF IMMUNODIFFUSION TESTS^{1,2}

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INTRODUCTION

Precipitin tests in which antigen and antibody diffuse through and react in semisolid matrixes have become indispensable tools in biochemical analyses since they were first introduced as such scarcely more than a dozen years ago. These tests can be categorized by technical characteristics as double-diffusion (DD), simple-diffusion (SD), or immunoelectrophoresis tests, and grouped collectively under the term "immunodiffusion."

No effort will be made in this essay to describe the various immunodiffusion techniques that have been invented, to list their many applications, or to review the latest publications appertaining to them; this will be done elsewhere (1). Rather, the purpose of this paper is to focus attention upon some of the more poorly understood technical and theoretical difficulties that may be encountered in these tests. Widely recognized and understood problems will be treated here only in passing, if at all.

An understanding of the mechanisms of diffusion in semisolid media and of antigen-antibody precipitation is necessary for adequately interpreting immunodiffusion tests. Hence, a short reiteration here of current salient information on diffusion and on antigen-antibody precipitation will be a helpful prelude to the discussion of problems that can affect interpretation of immunodiffusion test results.

INTRODUCTORY CONSIDERATIONS

Diffusion.—Diffusion of antigen and antibody through semisolid media conforms closely to the laws of gas diffusion (2, 3). Reactant diffusion consists of movement from an area of high concentration to areas of lower or no concentration. Presumably, this owes to thermal agitation of reactant molecules which causes them to collide with each other, with surrounding molecules or ions, and with their container walls in a frequency proportional to their concentration and to the surrounding temperature; these mutual collisions are the driving force of diffusion. The rate of reactant diffusion is regular and mathematically predictable (2). It increases as temperature is raised, as the semisolid medium's viscosity or structure "closeness," or both, is decreased, as the original reactant concentration increases, as certain immunologically nonspecific substances are added to it (see below), as its

¹ The survey of the literature pertaining to this review was concluded in January, 1960.

² The following abbreviations are used: DD (double diffusion); SD (simple diffusion).

affinity for the semisolid matrix decreases and, in immunodiffusion reactions, as the concentration of the substance with which it reacts specifically in the medium through which it is diffusing is lowered (2 to 5). Opposite conditions lower the reactant's diffusion rate.

Specific precipitation.—Specific precipitation is thought to occur in two stages (6, 7). In the first, antigen and antibody combine specifically and very rapidly upon being mixed. Then, more slowly, the original antigen-antibody complexes aggregate by any of several presently disputed but probably specific mechanisms to constitute a visible precipitate. The first stage of antigen-antibody precipitation seems to occur under broader experimental conditions than does the second (6, 7) but, with occasional exceptions (see below), both generally occur better in an environment approaching the natural body environment of antiserum.

Basically, there appear to be two types of precipitating antibody, which are classified by the kind of specific precipitate that they form and the proportions of antigen with which they can react (6, 8). One, commonly called "precipitating" antibody, classically is produced in rabbits and, hence, also is called "R" antibody. The other is "flocculating" antibody or, alternatively, "H" antibody since it is formed most readily by horses against most protein antigens. R antibody forms antigen-antibody complexes in a wide range of reactant ratios. Its precipitate is insoluble in excess antibody,³ and it has a variable, usually difficult solubility in excess antigen. H antibody, on the other hand, forms complexes within only a narrow range of antigen-antibody ratio, and these are readily soluble in either antigen or antibody excess. Antibody capable of reacting with antigen but not precipitating it can also be formed by animals in response to the injection of antigen (82). Such antibody may interfere with the activity of simultaneously present precipitating antibody, and may thus affect immunodiffusion results.

Some information obtained by classic precipitin tests in aqueous medium is not directly applicable to specific precipitation reactions in semisolid media because, in the latter, both reactants by diffusion are encountering each other in infinitely varied concentrations and react under dynamic conditions, while in the classic tests reactants are completely mixed simultaneously and have little margin for controlled interaction (9). Results of the two tests are most alike when that performed in an aqueous medium is

³ If regularly decreasing quantities of antigen are added to a series of tubes each containing the same amount of antibody, the quantity of precipitate produced in each tube progressively increases as the antigen concentration becomes lower and is maximal in a certain range of antigen-to-antibody commonly called the "optimal proportions" range. On the other side of this range, antibody becomes excessive as antigen is further diluted and the quantity of precipitate becomes progressively less. The optimal proportions range is bordered, then, by zones which for convenience are called "antigen excess" and "antibody excess." Similar observations and terms apply to tests in which decreasing quantities of antibody are added to constant quantities of antigen, although quantitatively the results from these two types of tests differ (6).

the "ring" or "interfacial" test, in which antigen is layered over antibody without mixing and precipitate forms at the interface. This test has enjoyed wide popularity because it is able to give visible precipitates over a much wider range of initial antigen-antibody ratios and hence is more useful as a rapid qualitative test than is the dilution precipitin test. The interfacial test owes its versatility to the fact that initially nonprecipitating antigen-antibody ratios are brought to precipitating ranges as the reactants mutually dilute themselves by diffusion across the interface. Actually, the ring test might be considered a primitive form of immunodiffusion. When this test is set up with antiserum incorporated in agar gel, it becomes an SD test, a technique of somewhat limited practical use but suited well to demonstrating principles of immunodiffusion and of antigen-antibody precipitation, as is attested by its wider use than DD in work dealing with problems of theory.

Immunodiffusion.—In the tube SD test, antigen is usually layered upon gelled antiserum. Reactant concentrations are arranged so that a large antigen excess exists, and the least amount of antibody is used which will give visible precipitate (2). Under these conditions, antigen must diffuse a short way through the antibody gel before it becomes dilute enough for available antibody to precipitate it. However, antigen continues to diffuse from its source so that no sooner does a precipitate zone form than it tends to dissolve at its rear as antigen accumulates in excess sufficient to reverse specific precipitation. By this dynamic process of antigen diffusion and reaction with newly encountered antibody, the front of precipitate appears to advance along with, but slightly behind, the front of diffusing antigen. Thus, precipitate band formation in SD tests is always an unsettled affair with the two reactants being matched in a continually changing spectrum of concentrations. This process is the same in all immunodiffusion tests except under conditions of exact antigen-antibody equivalence. In this condition, which often arises rapidly in DD test precipitin zones, antibody and antigen feed into an area to react under ideal conditions and proportions to give a stable, strong, non-moving band (3).

Factors which may affect immunodiffusion tests are numerous. Some will be mentioned here as background for further discussion. Intensity of precipitation is governed largely by the quantity of antibody available for reaction (2, 10 to 12), but also it is affected somewhat by antigen concentration (13), by salt concentration (14), and by nonantibody serum constituents (13) as well as by the length of time that a test is run (11, 13). The rate and direction of apparent precipitin band movement depend both upon the initial ratio of antigen to antibody and the ratio of diffusion coefficients of these two reactants. Generally, it moves toward the reactant of lesser concentration or diffusion coefficient, or both, with a speed proportional to the unbalance of both ratios. In the SD test employing very dilute antibody, the rate of movement of antigen is directly proportional to its absolute concentration and its molecular weight (2, 3) as well as to other factors mentioned above which accelerate diffusion.

Sudden changes in the diffusion rate of a reactant can cause "false" or secondary antigen-antibody precipitates or gaps in existing precipitate bands particularly in SD tests (15). Under certain experimental conditions, such as when moderately strong antibody is incorporated in gel in SD and a very strong antigen solution is allowed to diffuse into it, a number of visible precipitin bands will form, exceeding the number of antigen-antibody systems present (16).

Although SD and DD tests basically are the same (indeed, DD tests in which the initial reactant ratio is unbalanced become SD tests as reactions develop), the fact that in the latter both reactants must diffuse before precipitating makes these tests quite different in practice, and they often require different approaches to their use and interpretation. In the DD test, precipitation usually occurs with near-optimal reactant proportions, while in SD tests the original ratio by intention is quite uneven. This fact makes the former qualitatively superior and far less apt to form secondary precipitates because of reactant concentration changes (15). However, because bands move little if at all in DD, antigen-antibody precipitation characteristics, such as their reversibility, cannot be studied as conveniently as with the SD test. The two types of immunodiffusion techniques can be related and contrasted on the basis that the SD test magnifies events in specific precipitation which occur for only a very short time in the DD test when antigen and antibody fronts first converge upon and react with each other.

A special kind of DD test, immunoelectrophoresis, has unique problems in interpretation because it employs zone electrophoresis as well as diffusion to move reactants.

FACTORS AFFECTING THE INTERPRETATION OF IMMUNODIFFUSION TESTS

Diffusion.—Since antigen-antibody precipitation in semisolid media occurs while reactants or, perhaps, some of the smaller complexes formed by the reactants are actively diffusing, factors affecting diffusion must also affect immunodiffusion tests. The piecemeal, relatively slow, mixing and mutual dilution of reactants in these tests probably accounts for the important fact that an antigen-antibody ratio found at equivalence is likely not to correspond with the equivalence ratio obtained by a classic precipitin technique (9). In one recorded instance, for example, less antigen was required for equivalence with a given quantity of antibody in Oudin's SD test than in the classic method (9). This discrepancy is more readily understandable when one considers that optimal proportions in classic tests differ as differing absolute quantities of one reactant are mixed with a range of concentrations of the other (6). Discrepancies may be enlarged by differences in reactant diffusion coefficients (17), particularly in DD tests (3, 17, 18). Hence, equating directly the equivalence ratios obtained by tests in different media or even by SD and DD tests in one medium (15) is usually unreliable.

Another reason why immunodiffusion and classic techniques may yield divergent information concerning equivalence ratios is illustrated by a rather

unusual instance in which the classic method was found to provide a better resolution of the precipitin system. Under certain conditions, an antigen with two determinant groups can yield two optimal proportions peaks with its antiserum by classic precipitation when only one optimal proportion (i.e., precipitate band) may be found for the same system by immunodiffusion. The latter occurs because the antibody first reaching optimal proportions with one determinant group on the antigen molecule precipitates the antigen and prevents its movement to an area where its ratio with antibody to the second determinant would be compatible with precipitation (19). Thus, in such an instance, although antibody to the second determinant might diffuse through the precipitate formed by the first, it would be too dilute and its antigen too strong on that side of the existing precipitate to precipitate any antigen itself. Moreover, an immunodiffusion test can be susceptible to simultaneous reactions between antibodies to the second determinant, to the first, and to a combination of either or both and their carrier (20), so that one band and "optimal proportion" could be formed by three different kinds of antibody.

Factors affecting diffusion are likely to be particularly important in quantitative experiments. One should be able to determine the concentration of an unknown antigen by allowing it to react with its specific antibody in SD tubes, and comparing the speed of its downward diffusion, indicated by specific precipitate zone movement, with the speed of the same antigen used at known concentration. Similar measurements also should be useful for determining an antigen's diffusion coefficient or molecular weight (2). However, such rate measurements rarely can be translated simply into the desired data because they are so greatly affected by poorly understood conditions and substances which influence diffusion. For example, in the antigen-antibody reaction itself (21), at too low an antigen-antibody ratio, antigen does not diffuse freely since too much is consumed at the moving precipitate band front by reaction with antibody; moreover, antibody diffusion upward to replace that used at the front must be taken into account (21, 22). Antigen feeding freshly from its source into antibody-charged agar already traversed by antigen may react with existing dissolved or partially dissolved antigen-antibody complexes with consequent changes in its apparent diffusion rate (16, 21).

Effects of environment on reactants.—Interactions between the reactants and the supporting medium, or "inert" substances accompanying them in solution, and the effects of various substances upon antigen-antibody reactions must all be entertained as potential complications in interpreting immunodiffusion results. Antigens like lysozyme or fibrinogen tend to react with agar gel, falsifying apparent diffusion coefficient measurements (4, 23); chicken ovalbumin has been observed to do the same thing (24).

The diffusion coefficient of protein antigen through agar depends partly upon the influence of the pH of the gel upon the charge of the antigen (25). Generally, this coefficient is smallest when the charge of a protein is lowest.

The effect of pH seems to be rather small but, nevertheless, it could affect the results of a critical experiment that depends upon accurate reactant diffusion rate measurements. A somewhat different factor also dependent upon pH is a substance normally present in agar which tends to precipitate serum constituents at pH values less than 7, maximally at pH 5; fortunately, it is relatively inactive between pH 7 and pH 9 (25). The integrity of an antigen can depend upon pH. Some antigens may depolymerize at certain pH values into constituents that are distinct, not only physically but also antigenically, from the parent substance (26). Perhaps also controlled partly by pH is the tendency for antigens to form complexes; Williams & Grabar observed that serum albumin may complex with serum gamma-globulin (27). The possibility of interpretive errors caused by protein interactions is pointed out by Preer & Telfer (28). In addition to these effects, it is worth noting that at pH values exceeding 8.2 precipitation may be prevented, or precipitates may very readily dissolve. (4, 12). For this reason, most immunoelectrophoretic experiments are performed with buffers at pH not exceeding 8.2 (4).

The diffusion medium and various substances which it may contain influence immunodiffusion tests. In SD tests where interest focuses upon the apparent rate of precipitin zone movement, the effects of varying gel viscosity should be recognized (28). The concentration of gel influences its penetration by antigens considerably, a point which has been used to advantage in determining molecular weights (31). The viscosity and other properties of agar which impede antigen diffusion can depend upon its type and origin (25), how and to what degree it has been washed and purified (25), and how many times it has been subjected to the heating-gelling cycle (29). Its serum constituent-precipitating substance, mentioned above, can be removed by electrodialysis, but not very efficiently by ordinary dialysis (25). Neff & Becker in SD tests showed that chicken ovalbumin diffused more slowly through agar that had been melted and gelled twice than through agar that had been treated only once (29). Wieme (25) has emphasized the marked differences in electroendosmosis seen in agar gels made with agars of varying degrees of purity; such differences influence the migration patterns of serum constituents. Since these variations reflect large changes in gel charges, it is obvious that different agars dissolved and gelled according to a given method may not yield equivalent immunodiffusion results. Autoclaving agar can release substances which react with normal serum constituents (30).

The native inorganic salt content of agar, the type and concentration of salts employed in its solvent, and their effects on pH can be important in interpreting immunodiffusion results. For example, Wieme believes that the electroendosmotic properties of agar are governed by its sulfate content (25). Laboratory-grade agar that is sold in this country contains about 3.5 per cent of noncombustible material in which are found salts of copper, cadmium, iron, manganese, zinc, nickel, barium, calcium, strontium, and magnesium

(32). Two of these cations, cadmium and nickel, have been shown specifically to enhance precipitin reactions (32). The importance of agar impurities is illustrated by the observation that between 10 and 100 times as much cadmium salt is required for specific enhancement of antigen-antibody precipitation when deionized agar (0.01 per cent ash) replaces laboratory-grade agar (33). Furthermore, laboratory-grade agar contains sufficient electrolyte to support excellent precipitin reactions when it is made up in distilled (12) or deionized (33) water, and electrolytes have been removed from the solutions of reactants.

The enhancing effect of cadmium depends partly upon the solvent employed for the agar. With rabbit antisera, the effect occurs with barbital-buffered saline but not with unbuffered saline (34); with citrate-buffered saline cadmium is effective, but it is required in larger quantities, perhaps because of the chelating properties of citrate (33).

The electrolyte content of agar must be raised to about 10 times physiologic strength for optimal precipitation of some antigens by chicken antisera; at physiologic salt concentrations, precipitation may not occur at all (35, 36). Some antigens may become insoluble in simple salt solutions (37) or in certain types of buffer (38), creating the false illusion that they are absent from a crude mixture of antigens.

Miscellaneous substances may unpredictably influence reactant diffusion. In the SD reaction, Oudin showed that the addition either to the antiserum gel or to the antigen solution of glucose, polyvinyl-pyrrolidone, or snail hemolymph increased the apparent antigen diffusion rate without interfering with its regularity (39). Such nonspecific substances or even non-antibody fractions of an antiserum can also increase precipitin zone densities (13). The gamma globulin of rabbit antihuman serum albumin has been found to produce denser zones in SD when used with its albumin than when used alone (13). A method devised for identifying haptoglobin by its general property of coupling with hemoglobin (25) suggests complications when hemolyzed sera are employed in immunoelectrophoresis and immunodiffusion tests.

Experiments performed by Preer & Telfer explain an apparently sudden shift in antigen diffusion velocity, first observed by Oudin (39) in SD, when a nonspecific substance of high specific gravity is added in increasing quantities to a liquid antigen layer (28). Depletion of antigen at the interface by its diffusion into the antibody gel causes a local lowering of specific gravity; this initiates convection currents which keep the antigen mixed and virtually at its original concentration at the interface. In low-density antigen solutions, by contrast, there is no such mixing, and antigen becomes depleted locally without replacement except by diffusion from above, and its apparent diffusion coefficient is lower than when convection currents mix it. This difficulty is avoided by gelling the antigen as well as the antibody layer.

The unpredictability of effects caused by nonspecific substances is illustrated by other experiments of Preer & Telfer (28). These showed that su-

crose added to SD tubes at concentrations exceeding 0.2 *M* increased antigen diffusion velocity when the antigen moved down the sucrose gradient but decreased it if antigen diffused up the gradient; these effects were out of proportion to the influence of the sugar on medium viscosity or convection. Other sugars and inorganic salts had the same effects when the concentrations were high enough, but serum or its constituents did not.

Since the effects of various nonspecific substances at present are rather unpredictable, immunodiffusion experiments which may be affected by them can be controlled best by preparing reactant solutions in identical solvents [cf. (40, 41)].

Effects of other reactants.—The fact that nonspecific substances can influence apparent reactant diffusion rates raises the question of whether specific substances (i.e., reactants themselves) may have similar effects. This question is doubly important because from its answer might be obtained a better understanding of how a single antigen-antibody system can form more than one precipitate in immunodiffusion tests and, more generally, how specific precipitation occurs under the continually changing conditions of immunodiffusion. Examination of this question must be approached by the exposition of certain experimentally established facts which bear on it.

In SD a certain antigen A reacting with its specific antibody will form a precipitate band moving down the antibody gel at a predictable rate. If the concentration of A is increased, band migration will be proportionately faster, and if A is decreased, slower. If antigen B is added to antigen A, the rate of movement of band A will not be altered, even if it is migrating through agar containing not only anti-A but also anti-B antibodies (2, 3).

The converse situation of how one species of antibody may influence another has received some attention (42, 43) but too little to permit more than speculation. Since antibodies physically and chemically are the same as other gamma globulins, their diffusion rate might be thought to be regulated by the total quantity of gamma globulins (43), because the diffusion rate of a species of molecules is directly related to its total quantity (2). However, experiments performed by Jennings with different horse antitoxins in his triangle immunodiffusion test leads him to submit that antibody movement is influenced more by immunologically related than by merely physically related molecules (42); that is, nonantibody gamma globulins should have no effect upon the diffusion rate of an antibody gamma globulin.

Experiments reported by Buchanan-Davidson & Oudin (44) and by Jennings (45)⁴ show that, in general closely related antigen molecules can alter each other's penetration rates into an antiserum-charged gel while, as has been shown above, unrelated antigens do not affect each other. The same should hold for antibodies: if they are similar enough to cross-react they should not appear to diffuse independently, but they should be unaffected by other gamma globulins immunologically unrelated to them.

⁴ The author is grateful to Dr. R. K. Jennings for allowing him to read this and two other manuscripts before publication.

This situation may be simplified by considering each protein molecule in immunodiffusion as an independent agent being moved at random as it is jostled indiscriminately by surrounding particles, particularly those of low molecular weight. For one molecule being moved in the proper direction reaching a certain plane of the agar gel is merely a matter of time. If this hypothetical molecule is an antibody and it encounters an antigen at that plane, it will react with the antigen. If it is a nonantibody gamma globulin, an encounter with the antigen does not affect it. Now, if the initial number of antibody molecules is increased, the time that elapses before any antibody molecule is jostled across the interface decreases; the statistical likelihood that one of these molecules will be moved into this area is enhanced by the fact that many are being subjected to jostling rather than one. If this initial concentration of antibodies is mixed with ten times as many gamma globulin molecules, physically and chemically indistinguishable from the antibodies but unable to react with the antigen then, after a given time, several gamma globulin molecules may have been jostled across the interface; but since none can react with antigen there would be no evidence of this from the point of view of an immunodiffusion test. The chances for an antibody gamma globulin to move into the antigen layer remain the same as they were before nonantibody gamma globulin was added. Hence, the probability that one or any given number of antibody gamma globulins will reach a given plane in the antigen layer and reveal themselves by precipitating the antigen, should be related solely to their initial concentration and not to that of chemically or physically similar but immunologically distinct gamma globulins. Only if, by their increased concentration as indifferent molecules, these should raise the local "pressure" of all molecules rebounding from each other, could these added gamma globulins increase the rate of antibody diffusion. The experiments of Preer & Telfer (28) with antigens, however, suggest that serum protein molecules are too large to have a significant non-specific influence of this type.

Formation of multiple precipitates by a single antigen-antibody system.—A puzzling and interesting question regarding immunodiffusion is whether one antigen-antibody system can form more than one specific precipitin band in a semisolid medium. This question must be answered affirmatively in the face of available experimental data (12, 16, 17, 46 to 49);⁵ we are faced then with explaining how these multiple precipitates form, how they can be recognized, and whether this phenomenon can contribute toward elucidating

⁵ The proof that multiple lines can be formed by a single antigen-antibody system necessitates redefinition of the basic analytic assumption for immunodiffusion tests: that there are at least as many precipitin systems acting in a given test as there are visible precipitin bands (2, 3). This assumption seems to be justified only if reactants are used at close to the optimal combining ratios; if they are used out of balance, a special condition for SD in which, by design, this condition holds, the assumption is reasonable if one is diluted to the greatest degree compatible with visible precipitate formation.

antigen-antibody precipitin reactions. These multiple precipitates can be evoked easily in two ways, most readily in SD tests. One is to vary the temperature suddenly and widely during the development of the precipitin line, and the second is to employ a strongly unbalanced reactant ratio. Temperature-induced bands of precipitate commonly are called "striae"; gaps in a developing broad precipitate forming from the use of unbalanced reactant ratios are called periodic precipitates or Liesegang lines [named after a similar phenomenon described by R. E. Liesegang in 1896 as occurring with inorganic molecules (50)].

Incisive experiments on stria formation have been reported recently by Wilson (15). He showed that sharp temperature changes induce sudden variations in reactant diffusion rate, an effect particularly obvious in SD. From his findings, Wilson postulates that when a sudden antigen excess reaches the precipitation front as a result either of its concentration being increased in its depot or of having its diffusion rate accelerated by a temperature rise, it temporarily exceeds, locally, the concentration range within which antibody in the gel can precipitate it, and its front travels a short distance before becoming dilute enough to form a visible precipitate again. That is, it leaves a gap in the otherwise broad precipitate band moving down the tube. In the opposite situation, sudden slackening of antigen diffusion at the front should permit consolidation of the existing precipitate under conditions in which the antibody-antigen ratio remains in the precipitating range longer, so that when antigen excess again becomes sufficient to move the band front significantly, a stria (area of increased precipitate density) should be left behind. In Wilson's SD experiments, however, gaps were formed under these conditions instead of striae, presumably because diffusion of antibody toward the retarded antigen front left an area too depleted of antibody to form a visible precipitate later on, and consolidation of the precipitate was insignificant.

From his experiments, Wilson also noted that immunodiffusion tests were proportionally less sensitive to stria formation as higher molecular weight (more slowly diffusing) antigens were used, and that striae or gaps "always remained stationary and did not change in appearance with prolonged development" (15). From the first of these observations, it seems probable that the likelihood of stria or gap formation increases as the difference between diffusion coefficients of an antigen and its antibody increase, and that whether striae or gaps form depends both upon what kind of antibody is used and what its concentration is. The second observation has been made by others (2, 51) but, as Glenn was written,

"Conclusive evidence for a satisfactory hypothesis to explain why some workers believe that striae do not migrate has not been given. Although striae might not be subject to dissolution and reprecipitation as the minimal antigen concentration front progresses through the agar, this still does not satisfactorily eliminate the possibility that they may migrate with diffusing antigens" (14).

Stria formation raises interesting questions about specific precipitation and also helps to answer some. If a stria is composed of antigen-antibody precipitate, why does it presumably neither move nor dissolve when it is exposed to the antigen excess of the SD test? The answer seems to lie in the exclusive use of rabbit antibodies by those who have described this phenomenon; such antibodies form complexes poorly soluble in antigen excess. Thus, when a horse antihuman serum albumin system was used in the writer's laboratory, temperature-induced striae in SD tubes moved with the primary precipitate through the antibody gel, meanwhile slowly dissolving (16). In a single tube, this phenomenon could be repeated several times through its complete cycle of stria formation, movement, and dissolution. These striae were induced by suddenly lowering the temperature; if the temperature was raised abruptly, there was no change in the primary precipitin band other than an increase in its sharpness or compactness. This characteristic of compactness showed, incidentally, that the antibodies involved were of the flocculating rather than the precipitating kind, and formed antigen-antibody complexes readily soluble in quantities of antigen that they actually encountered (8, 52).

Liesegang or periodic antigen-antibody precipitation in its mechanics seems to be very similar to stria formation. This phenomenon will not occur when the antigen-antibody ratio is at or near balance initially in a test (12, 17, 46, 47), nor when one of the two chemicals forming a precipitate in an inorganic system does not sufficiently exceed the concentration of the other (50). In both antigen-antibody and inorganic precipitating systems, the weaker reactant must also be used at a certain suitable concentration. Thus, despite a large antigen-to-antibody excess, the SD test set up according to Oudin's original directions with low antibody concentration in the agar should not form visible Liesegang precipitates (2); but when a still larger antigen excess diffuses into less diluted antibody, periodic precipitates can form quite readily (16). Aside from general agreement that reactant concentrations critically govern periodic precipitation (12, 17, 46 to 50), one other observation finds common acceptance, i.e., that the concentration of the internal reactant (antibody in the usual SD) immediately adjoining the precipitate front on its side must be depleted (17, 47, 48, 50, 53). It must be low enough so that precipitation is interrupted, and the external reactant becomes able to diffuse through both the precipitate and the internal reactant-depleted area where it exceeds the capacity of the remaining internal reactant to precipitate it. Presumably, by further diffusion, the external reactant dilutes itself while moving into the range of less depleted internal reactant, and the process of precipitation begins anew.

The conditions reported to be necessary for periodic antigen-antibody precipitation (16, 17, 46 to 49, 54) generally agree with those required for eliciting Liesegang rings (50, 53). Large disproportions in reactant ratios have been used in all instances, and local reactant depletion has been postu-

lated in several (16, 17, 47, 48). How such depletion can occur has been demonstrated recently by the writer (83). Like striae, Liesegang lines often do not appear to move (3, 17, 55), but they can do so when flocculating-type antibodies are used (16, 47). Such band movement requires a dynamic process in which precipitation and its reversal are occurring continuously before, through, and behind the precipitate. This explains why it is unlikely to occur with antigen-antibody systems forming complexes not rapidly soluble in reactant excesses.

Explanations other than the Liesegang phenomenon have been offered for multiple precipitate bands formed by supposedly single antigen-antibody systems (46, 48, 56). However, in the end these usually depend upon some notion of either antigen or antibody heterogeneity, and hence beg the question of how a homogeneous system could produce multiple precipitates. They serve, rather, to emphasize the problems of reactant heterogeneity. Basically, they must assume heterogeneity of determinants in a species of antigen molecules and a corresponding heterogeneity of antibodies formed in response to such an antigen. If such a system is tested in DD, the following example may illustrate the course of events: Antigen A:xy (x and y being hypothetical determinants on carrier A) reaches an optimal precipitation ratio with antibody to A:x; anti-A:y is present but may be too concentrated in this area to accompany anti-A:x by precipitating the antigen through its y determinant.⁶ Hence, it diffuses through the anti-A:x precipitate, becoming more diluted until on the other side it reaches precipitating proportions with the antigen and its y determinant. Since all antigen now is being precipitated by anti-A:y, antibody to A:x no longer is dynamically held behind the first precipitate that it formed, and diffuses through it perhaps to precipitate anti-A:y-A:y soluble complexes formed in antibody excess or to add to the precipitate being formed by the A:y system. According to this hypothesis, at least as many precipitates possibly could form as there are antibody species directed against individual antigenic determinants. This idea has been developed from the work and thoughts of several experimenters (20, 45, 46, 48, 56) and can be viewed with many complicating variations such as whether flocculating or precipitating antibody is used, whether an antigen polymerizes or depolymerizes during a test (26), whether antigen exists in such forms as A, A:x, A:y, and A:xy, or whether this and the Liesegang phenomenon occur simultaneously. Careful analysis of these possibilities in connection with the conditions of a test and its reactants is necessary in order to offer an explanation for any given instance of multiple precipitate formation from a supposedly homogeneous antigen-antibody system.

The practical questions of how to avoid or recognize striae or periodic

⁶ Antibody to the carrier itself could be treated hypothetically together with the other two species of antibody and in the same manner, but will not be done here for the sake of simplicity.

precipitates can be answered on the basis of observations discussed above. These artifacts can be prevented from forming by employing reactants in equivalent concentrations and by avoiding conditions which may suddenly alter reactant diffusion rates. In SD tests, where reactants are meant to be used at disproportionate concentrations, visible Liesegang bands can be kept from forming by using very dilute internal reactant. If they should form, these secondary precipitates usually are recognizable by their immobility when R-type antisera are used. Striae produced by H-type antisera are likely to dissolve after a short time, but the Liesegang rings that these sera produce may not be sufficiently different from primary precipitates for differentiation. Perhaps the most foolproof immunodiffusion test for avoiding or readily recognizing secondary precipitates is one of the variations (3, 57), in which antigen and antibody are made to diffuse across each other with an appropriate arrangement of reactant depots at a 90° angle or less, since, in one of these tests, effects produced by sudden changes in reaction concentration or by overwhelming reactant excesses identify themselves by their influences on the direction of precipitin line growth, i.e., its angle. The tip of this line always reflects the state of antigen-antibody ratio at any given time, forming as it does at the optimal proportions ratio.

Antibody heterogeneity.—The above discussion brings out a fact widely recognized but often overlooked in practice, namely, that antibodies to a given antigen are discouragingly heterogeneous, as is responsiveness itself to antigens. Thus, antigen may provoke formation of no antibody, antibody of one kind, or antibody of several kinds (30, 58, 59). One horse may form flocculating and another nonprecipitating antibodies (60); or one can form precipitating or flocculating antibodies (60, 61), depending upon the vaccination procedure or the type of antigen injected (61). Human autoantibodies to thyroglobulin can be either of precipitating or flocculating type (62). Usually, precipitating antibodies are gamma globulins, but they can be found in various animals and man in one or more of the gamma, beta, or alpha globulins, depending upon the antigen (6, 61, 68) and how it is used (61, 63 to 69). The molecular weights of antibodies can vary considerably (6, 7, 66, 68, 70, 71). These observations caution against equating results obtained with different antisera against one antigen even if these are from one species of animal, or obtained from the same animal at different bleedings; against pooling antisera without first learning something about their antibodies; and against assuming that a gamma globulin fraction represents all the antibody to be found in a serum.

Even though animals of one species may manufacture only R-type antibodies to an antigen, antigen-antibody complexes formed by different serum samples can vary in their solubility in antigen excess (72). Thus, the type of precipitate zone produced in an immunodiffusion test can differ from one serum to another (52). Antibodies produced by a single animal may have varying specificities (73).

Horses and other animals hyperimmunized with human serum give cross

reactions with the serum components of many other mammals, or even of fowl (74, 75), suggesting that hyperimmunization causes the formation of antibodies able to react more avidly (4) and against more determinant groups on an antigen (58) than antibodies provoked by moderate immunization. Thus, hyperimmune antibodies are more sensitive in detecting individual constituents of antigen but also are more likely to react with similar constituents in different antigen mixtures (75, 76). Weak immunization, by contrast, has been found to yield reliably species-specific antisera for detecting human serum components in DD tests (75). For detecting antigenic components of a serum, antibodies with maximum species specificity can be obtained by using as antibody producer the species taxonomically most closely related to that supplying the antigen, e.g., the mouse and the rat (77). If this is impracticable, however, preliminary experiments may reveal that one animal species produces more specific antiserum for a given antigen than another (78, 79). In some instances, it seems practicable to use animals in the same species for immunodiffusion tests [cf. (80)].

Improper use of the proper antibody may lead to incorrect conclusions. For example, a false reaction of partial identity can be obtained between two unrelated antigens if they are compared through the use of an antiserum prepared against a third antigen related to both (20). Ideally, two antigens should be compared using a mixture of antisera against both (20). False reactions of identity can occur if the pattern of reactant depots, or the original reactant concentrations, do not permit sufficient diffusion into the area where bands may cross or join (81). On the other hand, large differences in adjoining depots of identical antigen in the presence of certain concentration gradients of antibody can yield false reactions of nonidentity (20, 48). In this case, one of the antigens may be exhausted before the other while antibody is still diffusing.

A specialized type of immunodiffusion of unsurpassed analytic value for characterizing biologic fluid macromolecules is immunoelectrophoresis. It is impossible, here, to devote more than token space to this technique because, although factors which govern other immunodiffusion tests also govern its second stage, those operating on its first stage are different and fall within the realm of zone electrophoresis. For expositions of factors affecting zone electrophoresis as it is used in immunoelectrophoresis, the writer recommends Grabar's recent instructive review (4) and Wieme's excellent monograph on agar electrophoresis (25).

Immunodiffusion tests have served their careful users well in the past, enabling them to accomplish analytic feats which would have astounded the immunologist of 20 years ago. Their usefulness in the future will be limited only by the imagination of those who apply them, and by the discrimination and understanding which which they are utilized.

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IN VITRO CELL-VIRUS RELATIONSHIPS RESULTING IN CELL DEATH^{1,2}

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INTRODUCTION

The title of this review might be interpreted in several ways. The present reviewer chooses to use it to consider data available from cell culture systems concerning several circumstances under which viruses cause severe damage to cells. It will be necessary to interpret "cell death" loosely for, in some of the experimental work reviewed, there is no proof that the observed viral-induced alterations in cells proceed to complete cell destruction. Throughout the review we shall, in fact, frequently accept the morphologic alterations in cells referred to as cytopathic effect (CPE) as an indication of cell damage, regardless of the fact that not everyone may agree as to what these changes imply in regard to cell function or to cell survival.

To the virologist it is a common experience to see cell damage associated with viral infection. It is, in fact, his most important means of recognizing the activity of viral agents and, except for relatively few viruses, he would be at a loss for a means of assaying and characterizing viruses were it not for the tendency of viruses to damage and destroy cells. The capacity for damaging cells and thus altering host tissue and organ function to result in what we recognize as disease is such a frequent characteristic of viruses that it has often been included in the effort to provide a definition of a virus (1, 2). As more has been learned about viruses, however, and as more subtle means of recognizing them have developed, it has become evident that sometimes animal viruses, as well as bacterial, insect, and plant viruses, may exist and multiply in cells without producing overt damage or cell death (3, 4). Since, to the virologist who is primarily interested in prevention and control of disease, the damaging effect of viruses on cells is the very nucleus of his problem, it then becomes important to learn more about the conditions under which viral infection often destroys the host cell and the circumstances in which it may not.

¹ The survey of the literature pertaining to this review was concluded in January, 1960.

² The following abbreviations will be used: CPE (cytopathic effect); DNA (deoxyribonucleic acid); FPV (fowl plague virus); NDV (Newcastle disease virus); RNA (ribonucleic acid).

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On looking through the literature concerned with the effects of animal viruses on cells it becomes apparent that investigators usually have been very cautious in expressing their views as to the causative factor in the virus-cell relationship that results in damage to the cell. Opinions have been couched in the most general of terms. This caution seems still to be justified because animal virology is only now entering the era of biochemical comparison of the infected with the normal cell that may eventually provide detailed answers. There has accumulated, nonetheless, considerable data relative to several phenomena that will have to be accounted for by any ultimate biochemical explanations of cell damage. This review will consider the main outlines of these phenomena as seen in *in vitro* systems.

THE RELATIONSHIP OF CELL DAMAGE TO PRODUCTION AND RELEASE OF INFECTIOUS VIRUS PARTICLES

There have been many reports in which investigators have indicated that the introduction into cell cultures of a particular virus was followed by the onset of obvious cell damage and that, during the general period of cell degeneration, viral increase was detectable in the cells or in the culture medium. Such studies have been reviewed by Enders (5), and Lynn & Morgan (6). Kinetic studies utilizing single-cycle infections of cells to examine more closely the time relationships between viral multiplication and cellular alterations have been much fewer, however, and it appears appropriate to summarize some of these.

Poliovirus.—When tube or bottle cultures of the HeLa strain of human carcinoma cells or of monkey kidney cells are inoculated with large quantities of virus in order to infect all cells as simultaneously as possible, a regular sequence of events follows (7 to 11). Release of new virus into the fluid begins at about the sixth or seventh hour after infection and proceeds over several hours. Most of the virus yield is released by about 12 to 14 hr. The first abnormalities in the cell visible by phase-contrast microscopy or in fixed and stained cells are seen at 3 to 4 hr. These early changes are variously described, but they include loss of chromatin in the central zone of the nucleus (11) and the appearance of a cytoplasmic mass (9). Abnormalities including distortion of the nucleus, wrinkling of the nuclear membrane, displacement of the nucleus, rounding and contraction of the cell are progressive in their development until the cells leave the glass and appear to disintegrate at about 24 hr. after initiation of the infection.

The course of events described is similar for cell strain HeLa, human fetal lung cells, and for monkey kidney cells, and occurs with all three types of poliovirus. In human amnion cells the pattern of CPE is somewhat different and virus release does not begin until about 10 hr., but signs of cell damage are visible at 8 hr. and virus release seems to occur as certain stages of CPE are reached (12).

It is notable that virus release occurs at a time when there is marked cell damage but that distinct CPE is visible for 2 to 3 hr. before the beginning

of virus release. Sequential studies by Reissig *et al.* (11) and by Oddo (13) correlating the appearance of intracellular virus, extracellular virus, and CPE give us information important to this point. In the experiments of these workers, intracellular infectious virus was first detectable at about 4 to 5 hr. after infection and rapidly increased to a peak level at about 8 hr. Virus release was first detectable at about 6 hr. After peak intracellular levels were reached at 8 to 10 hr., intracellular virus declined gradually as extracellular levels increased. This sequence of events in the one-cycle growth curve in HeLa and monkey kidney cells has been confirmed by several investigators (14 to 18). Reissig *et al.* (11) found that the time of appearance of the infectious virus within the cell was usually coincident with definite changes in the nucleus characterized by condensation of chromatin-like material about the nuclear membrane and the appearance of two intranuclear inclusions. As intracellular accumulation of virus proceeded there was a steady increase in CPE, and by the time virus was released the cells showed signs of advanced damage.

The intracellular changes associated with poliovirus multiplication have been extended to electron microscopic detail by Kallman *et al.* (19). In monkey kidney cells it was found that the first morphologic changes that seemed related to viral infection were seen at about the time that release of infectious virus from the cell began, and seemed to represent a stage more advanced than the earliest changes visible by light microscopy. Although dense bodies larger than mature virus particles were present in the cytoplasm during the time that virus release was in log phase, no particles resembling mature virus were visible at any time. At the end of the virus release period cells were rounded, the nucleus distorted, mitochondria were swollen, and there were vacuoles in the cytoplasm.

Studies with single infected cells isolated in droplets under oil provide additional detail to relate CPE to virus production and release. When such cells are followed and their virus yield is measured at frequent intervals (20, 21) it becomes evident that, although in large populations of cells release of poliovirus appears to take place over several hours, it is actually released from individual cells within a period of only about 30 min. It is not possible to follow intracellular viral production and viral release in the same cell, so that observations on signs of damage are oriented around virus release, but Lwoff *et al.* were able to follow effects on the cell by phase-contrast microscopy and to relate these effects to the time of virus release. About one hour before virus release, cells were seen to contract into a rounded shape, and granular material tended to concentrate in the nuclear region leaving a hyaline zone at the periphery of the cell. At the time of virus release the hyaline zone became vacuolized and irregular, giving the impression that the hyaline zone had dissolved and that virus release occurred through partial lysis of the cell. Howes (21) confirmed the abrupt nature of virus release but found that the time of release was quite variable and that there appeared to be a period of intracellular retention of mature virus prior to release.

Studies following intracellular antigen production by means of fluorescent antibody confirm the parallel development of intracellular viral material and cytopathic changes (13, 22, 23).

Adenoviruses.—The virus growth curve and development of CPE associated with adenovirus infection of HeLa cells have been studied in several laboratories (24 to 27). The details have varied somewhat from study to study, for infecting large numbers of cells simultaneously with these viruses is difficult and their assay is less precise than for poliovirus, but all agree on the relationships between CPE and virus multiplication.

The first evidence of new viral material in the cell is the appearance in the nucleus of antigen identifiable by fluorescent antibody (27). This makes its appearance about 12 hr. after the initiation of infection and steadily increases in concentration. Antigen also gradually appears in the cytoplasm between 12 and 24 hr. At approximately 16 hr. infectious virus is first detectable within the cell. Intracellular virus undergoes a very prolonged rise, reaching peak levels sometime after 25 hr. In monolayer cultures, exit of the virus from the cell appears to be very inefficient and slow (26).

Visible changes in the cells that are seen in fixed and stained preparations make their first appearance in the nucleus at 12 hr. after infection at just about the same time that virus-specific antigen is first recognizable. Nuclear alterations are distinct by the time measurable levels of infectious virus appear at 16 hr., and thereafter increase relentlessly. It is noteworthy that with these viruses morphologic signs of cell damage are particularly prominent in the nucleus. Intranuclear crystal-like inclusions can sometimes be seen. It seems quite clear from studies employing fluorescent antibody (27), and from the electron microscopic observations (28 to 31) of large crystalline arrays of virus particles in the nucleus, that the primary site of virus production is in the nucleus.

Herpes B virus.—Reissig & Melnick (32) followed the development of herpes B virus in monkey kidney cells with light microscopy, electron microscopy, and measurement of extracellular virus. From 4 to 6 hr. after inoculation, cells began to show nuclear changes consisting of increase in volume and alteration of shape. At 6 to 10 hr. the central zone of the nucleus became clear, the nucleolus disappeared, and the chromatin condensed at the periphery. Margination of the chromatin became most pronounced from 10 to 14 hr., at which time dense round particles, presumed to be virus particles, appeared in this chromatin. At about the same time, virus particles were evident in the cytoplasm and on the external surface of some cells, and infectious virus became detectable in the fluid medium. At this time obvious destruction of cells was not apparent and it seemed that some virus was excreted for a few hours before cell disintegration became visible in the histological preparations. Further increase in numbers of intracellular particles and extracellular infectious virus was associated with progressive degeneration of the cells.

Additional studies indicating a generally parallel course of production

of infectious virus and appearance of CPE might be cited but this would only belabor the point. The examples already discussed seem clearly to document what most virologists will be willing to accept anyway, that in the usual multiplication cycle of cytotoxic viruses, development of recognizable morphologic abnormalities of the cell is closely correlated in time with intracellular viral accumulation and with release of infectious particles. This brings us to the question of whether or not cell damage is dependent upon production of infectious particles or upon excretion of such particles.

Dissociation of CPE from infectious virus production and release.—Pursuing data concerned with poliovirus as an example of the effect of viruses on cells, evidence can be found indicating that CPE occurs even though virus release is prevented. Larson *et al.* (33) report that if poliovirus-infected HeLa cells are incubated at 25°C. instead of at 37°C., intracellular accumulation of infectious virus proceeds at a rate similar to that at 37°C., but release of virus is inhibited. Very little extracellular virus is detectable by 24 hr. after infection but at that time the cells show what was described as typical cytopathology. Roizman (34) has confirmed the finding that large quantities of poliovirus are accumulated in cells at 26° to 32°C. without evidence of release for 18 to 20 hr. but reported no observations on the effect on the cells.

Additional evidence is provided by studies by Ackermann *et al.* (10) who demonstrated that an appropriate amount of fluorophenylalanine added to the medium of HeLa cells within 3 hr. after infection with poliovirus sharply inhibited release of infectious virus. Development of characteristic signs of cell degeneration, however, proceeded at the usual rate. These workers did not study intracellular virus so that we do not know whether or not CPE was related to intracellular accumulation of either infectious or non-infectious viral components. This point is of considerable importance for, in a good many virus-cell systems that we shall cite, production of virus-specific material accompanies cell damage even though release of infectious virus does not occur.

Proceeding from related work on the use of proflavine for inhibition of phage synthesis (35 to 37), Ledinko (38) has found that the addition of proflavine to HeLa cell cultures immediately after infection with poliovirus almost completely inhibits production of infectious virus, but the cells undergo degeneration at the time that would be expected in an uninhibited culture. Upon cell disintegration non-infectious virus particles are released into the medium. These non-infectious particles occur in the same general number as produced by control cultures, and by electron microscopy appear similar to mature virus particles. Complement-fixing antigen in amounts similar to that found in control infected cultures accumulates in proflavine-inhibited cells and escapes when the cells disintegrate. Somewhat similar data have been reported by Brown & Stewart from studies of foot-and-mouth disease virus in pig kidney cells (39). Proflavine added immediately after infection of cells prevented production of infectious virus but cells underwent typical degeneration. Brown & Stewart found that although the production of in-

fective particles was inhibited, if the cultures were extracted with phenol, infectious RNA could be demonstrated at a level approaching that of control infected cells. This, plus the fact that complement-fixing antigens were below detectable levels in the yield of proflavine-inhibited cells, led them to conclude that in this cell-virus system proflavine inhibited synthesis of viral protein components much more severely than it did the synthesis of viral RNA.

From the foregoing studies indicating that cell degeneration not recognizably different from that associated with the normal virus cycle occurs under circumstances in which there is neither accumulation nor release of infectious particles, it may reasonably be concluded that cellular damage is not dependent upon the infectious quality of the virus. It appears, also, that whatever the cellular lesion may be that results in cell destruction, its effects are being exerted much before the time of release of viral products. More information related to these points will be included in the following sections.

THE CYTOPATHIC EFFECT OF MASSIVE VIRAL INOCULA

There have been several demonstrations of acute, non-transmissible, cytopathic effects on cells in culture following inoculation of the cultures with very large quantities of virus. These have, so far, been limited almost entirely to the myxoviruses. The first study that provided insight into the processes involved was that of Henle *et al.* (40) and their findings provide a general description of the phenomenon.

If HeLa cells are inoculated with small amounts of standard egg-adapted laboratory strains of influenza virus, there is no cytopathic effect and no production of new infectious virus. The cells appear to be resistant to infection by influenza virus. If, however, the virus is added in large quantities, then within 12 to 96 hr., depending upon the concentration of the virus, the cells become granular, rounded, shrunken, and finally apparently disintegrate. This effect is neutralized by immune serum, and the cytopathic activity cannot be separated from the virus particle. Fully infectious virus is most effective but with careful exposure to heat or to ultraviolet light, virus suspensions lose infectivity at a faster rate than they lose their cytopathic effect. An important point is that Henle and his co-workers showed that although new infectious virus is not produced in the cells, there is, nevertheless, extensive production of viral material measurable by hemagglutination and by complement-fixation. In their experiments production of such viral components was well under way in cells before obvious cell damage became apparent. In a typical sequence soluble S antigen was detectable in the cells at 4 hr. after exposure to virus, and the complement-fixing V antigen and hemagglutinin were detectable by 5 hr. These materials reached their maximum in the cells at about 12 hr. at which time CPE became well developed. Virus-specific materials did not appear in the medium until cells began to disintegrate at about 24 hr.

The production of viral components in this influenza-HeLa cell system

is considerable in quantity. Henle *et al.* calculated from their hemagglutinin titer data that the production of hemagglutinin units per cell was roughly equivalent to that produced in a standard cycle of influenza multiplication in the chicken embryo allantois. It appears, therefore, that in many respects, including the time sequence of the intracellular cycle and the total quantity of virus-specific material produced, influenza virus multiplication in HeLa cells resembles the cycle seen in cells of the allantois, except that the HeLa cell does not produce particles that are infectious and that virus escapes the HeLa cell only upon cellular disintegration.

In the work of Henle *et al.* it appeared that an infecting dose of virus of 10 egg-infectious units or more per cell was required to produce a clear-cut cytopathic effect. This implied that a high multiplicity of infection might be a requirement for the initiation of the cycle in HeLa cells. More recently, Wilcox (41, 42) has studied a similar cytopathic effect of Newcastle disease virus (NDV) upon Earle's L cell line of mouse fibroblasts. His basic findings were like those of the influenza-HeLa cell system except that he was able to demonstrate that, in addition to much non-infectious hemagglutinin produced in the cultures, 10 to 20 per cent of the cell population did yield very small numbers of infectious particles (2 to 3 PFU/cell). In addition, in this system employing NDV that allows use of the sensitive plaque-assay of infectious particles, it appeared that only one plaque-forming unit per cell was sufficient to initiate the abortive cycle. It seems probable that this applies to the influenza-HeLa cell system also, since the virus-cell ratios employed by Henle *et al.* were only of the general order required for myxoviruses to cause infection of most of the cells of a monolayer culture in a short adsorption period. The important point is that a large proportion of the cells must be infected and undergo degeneration in one cycle for the CPE to be seen, because the yield from the first cycle of infection is not able to infect new cells and thus cause a progressive infection of the culture.

Further insight into this abortive cycle of myxoviruses in relatively resistant cells has been provided by study of fowl plague virus (FPV) in L cells by Franklin & Breitenfeld (43). Again, this cell appears incapable of supporting a cycle that yields complete, fully infectious FPV, but there is intracellular production of non-infectious hemagglutinin and complement-fixing S antigen in quantities and in time sequence almost identical to those of the cycle in fully susceptible chick embryo cells. The yield of viral material is retained in the cell until the cellular degeneration that accompanies the cycle reaches the point of cellular disintegration. When Franklin & Breitenfeld followed the course of the cycle in L cells using specific fluorescent antibody against the S antigen and against the hemagglutinin component, they found that the S antigen appeared in the nucleus and hemagglutinin appeared in the cytoplasm as in the normal FPV cycle. The migration of S antigen from the nucleus into the cytoplasm to unite with the hemagglutinin component did not take place, however. The S antigen is apparently confined in the nucleus and the resulting viral yield is non-infectious, presumably be-

cause of deficiency of the nucleoprotein component ordinarily contributed by the S antigen.

Influenza virus will produce an incomplete, abortive cycle with cellular destruction in several human cell lines (44), and it appears that others of the myxoviruses will also do this in certain cell lines. Sendai virus in the MCN line of cells (45), in HeLa cells (45, 46), and in chicken embryo lung cells (46) yields large amounts of non-infectious hemagglutinin. Large inocula of egg-adapted mumps virus have been shown to produce a rapid cytopathic effect without yielding significant infectious virus, but details of the cycle are not available (44, 46 to 48). The extremely rapid effect (3 to 6 hr.) of relatively slowly multiplying mumps virus caused Henle *et al.* (47) and Russel & Morgan (48) to suggest that the CPE may be related to the enzyme of this virus that causes hemolysis of erythrocytes. Sendai virus and NDV possess vigorous hemolysins also, however, and the demonstration that the rapid CPE caused by these viruses is associated with an abortive cycle suggests that for mumps virus the underlying mechanism may also be an abortive cycle, and that the processes leading to CPE are triggered very early in the reproduction process.

Study of these abortive cycles should continue to produce important information pertinent to the multiplication and cell damaging effects of the myxoviruses. It seems certain that more than one pattern of abortive cycle will become evident, for it has been found that for mumps, Sendai, and Newcastle disease viruses there seems not to be a nuclear phase of S antigen production in the normal cycle (46). Furthermore, even though influenza virus, like fowl plague virus, normally has a nuclear phase of S antigen production, in an abortive cycle in human conjunctiva cells the nuclear phase seems to be lacking, in sharp contrast to the findings in Franklin & Breitenfeld's study of the abortive cycle of fowl plague virus (46).

In addition to the myxoviruses, vaccinia virus has been reported to cause a rapid, cytopathic effect when large inocula are added to cultures of embryonic mouse lung (49) or to cultures of mouse leucocytes (50). Here, too, the cytopathic activity cannot be separated from the infectious virus in the inoculum, and the activity is neutralized by specific antiserum. The cells undergo degeneration, but do not yield infectious virus. Whether or not they produce viral components other than complete infectious virus has not been investigated, but this information will be essential to further understanding of the reaction.

Relationship of in vitro studies to in vivo viral toxicity.—Although this is not the place for an extensive discussion of the *in vivo* phenomenon of viral toxicity [cf. (51)], it seems appropriate to point out that the rapid cytopathic effect of large viral inocula in cell cultures has major features in common with *in vivo* reactions and that, at least in the cases of the myxoviruses, most of the *in vivo* reactions may well have their bases in similar incomplete or abortive cycles within cells to which the virus is not fully adapted. An aberrant cycle of growth was demonstrated for influenza virus in mouse brain by

Schlesinger (52), and in the mouse lung by Ginsberg (53), although the latter has interpreted his data somewhat differently. An abnormal multiplication cycle can be demonstrated in the mouse liver after intravenous inoculation of influenza virus (54). Although it has not been possible by the use of complement-fixation and hemagglutination techniques to demonstrate the production of non-infectious viral materials associated with the pneumonia developed in mice after intranasal instillation of Newcastle disease virus (55, 56), recent studies of Prince & Ginsberg (57 to 59) with NDV in Ehrlich ascites tumor cells suggest that this may not exclude the possibility that large quantities of viral material are produced. These workers found that NDV in its destructive effect on Ehrlich ascites tumor cells in the mouse did not produce viral components that could be detected by infectivity, hemagglutination, or complement-fixation. They were able, however, to demonstrate by use of fluorescent antibody that large quantities of intracellular antigen appeared in the cells subsequent to infection.

CELL DAMAGE PRODUCED BY TOXIN-LIKE MATERIALS SEPARABLE FROM INFECTIOUS VIRUS PARTICLES

Rapidly produced cytopathic effects of a distinctly different nature from those just discussed have been studied in several laboratories. These studies have been concerned with cytotoxic materials that appear in virus infected tissue cultures but are separable from the infectious virus particles. These materials have been found associated with two viruses, poliovirus and adenovirus, but the cytotoxins found with these two viruses appear to be different in several respects. That associated with poliovirus will be described first. For the sake of simplicity, in subsequent discussion these materials will be referred to as cytotoxins or toxins even though their exact nature is not yet known.

Cytotoxin associated with poliovirus.—Ackermann and co-workers have described a rapidly developing cytopathic effect observed when low dilutions of type I poliomyelitis-infected HeLa cell culture fluids are added to test cultures of HeLa cells (60 to 62). Within 2 hr. many cells contract, separate from the glass, and appear to lyse. This effect is distinct in time from the later CPE that appears with poliovirus multiplication in the cells. The toxic component is much less stable to storage than the infectious virus particle, being largely inactivated in 2 weeks at 4°C. Antiserum produced by immunizing monkeys with infected HeLa cell homogenates neutralizes both the rapid cytopathic effect and viral infectivity, but antiserum from animals immunized with infected monkey spinal cord has a high degree of neutralizing effect on viral infectivity and none on the rapid cytopathic effect. Antiserum produced against uninfected HeLa cells has no antitoxin activity, yet uninfected HeLa cells will absorb the antitoxin from antiserum produced against infected HeLa cell homogenates. This suggested to Ackermann *et al.* (62) that the toxin is distantly related antigenically to a component of HeLa cells, and is without antigenic relationship to components of the virus. It is apparent

that additional study will be needed to clarify the status of this material and its relationship to the virus and to the cell.

Cytotoxin associated with adenoviruses.—Considerably more information is available about the cytotoxin that appears in adenovirus infected cultures. This material has been studied in several laboratories and, while they do not agree completely on all points, they seem to be studying the same factor and their major findings are in general agreement.

It is evident that when certain types of adenoviruses multiply in HeLa cells a factor is produced that has a rapid cytopathic effect when transferred in low dilution to other HeLa cell cultures. This has been demonstrated by Pereira & Kelly (63), Pereira (64), Everett & Ginsberg (65), and Rowe *et al.* (66). This factor is produced by many adenovirus strains but most abundantly by types 1, 2, and 5 (66), and most study has been of the material produced by type 5. Its effect can be demonstrated on the HeLa and KB lines of human cells and on serially cultured monkey heart cells. The effect on cells is seen particularly as rounding and clumping, and the cells can more readily be shaken loose from the glass. This effect occurs from one-half to 4 hr. after cells are exposed to the toxin and, therefore, is considerably separated in time from the later CPE that appears with viral multiplication. The CPE of the toxin is also distinguished from that which accompanies viral multiplication by the fact that the early effect does not produce the characteristic nuclear alteration seen with adenovirus infection (64, 65). An important point made by Pereira (64) is that although the toxin causes marked rounding and clumping of cells, it does not destroy them and seems not to inhibit their division.

The toxic component in adenovirus cultures is readily separated from the infectious virus particles because it sediments more slowly in the centrifuge (64 to 66). In addition, it is more stable to heat than is the infectious particle. The activity of the toxin is not destroyed by 60°C. but is by 70°C. for 30 min. (66); it is not affected by diethylether; it is not dialysable; it is destroyed by trypsin, but it is not destroyed by ribonuclease or by deoxyribonuclease (64 to 66). These characteristics certainly suggest that the activity of the factor resides in a protein component. Wilcox & Ginsberg, however, in a brief report (67) have characterized it as a ribonucleoprotein and state that dissociation of the RNA and protein components by cationic exchange resin results in loss of toxic activity.

The rapid cytotoxic activity of a preparation is neutralizable by convalescent human sera and rabbit antiserum, but the antitoxic potency of a serum does not bear a constant relationship to either its infectivity neutralizing or its complement-fixing antibody titer (66). The antigenic relationships of the toxic factor appear to have been clarified considerably by recent fractionation and purification studies by Pereira *et al.* (68) and Klempner & Pereira (69) on the soluble antigens associated with types 2 and 5 adenoviruses. These workers found the toxin to be one of three soluble complement-fixing antigens, accounting, however, for only a small portion of the

total complement-fixing antigen produced in adenovirus infected cells. It appeared, in addition, that there was an antigenic relationship between toxic factor produced in type 2 and type 5 virus infected cultures.

What part these extraviral toxin-like materials may play in viral infections is yet to be determined. In cultures infected with adenovirus the cytotoxin becomes detectable within cells at the time that complement-fixing antigen first becomes measurable about 20 hr. after infection (27). This is about 4 hr. after the intracellular appearance of new infectious virus, and after CPE is already evident. No studies have yet been reported relating the cytotoxin from poliovirus cultures to stages of the poliovirus reproductive cycle. Regardless of whether or not a purposeful role is found for these materials in the virus cycle, it appears that they may contribute to the CPE seen in cell cultures. It is noteworthy, however, that not all type I poliovirus strains stimulate formation of detectable amounts of cytotoxin, even though they grow to equal titers and produce comparable cell destruction. Indeed, Ackermann *et al.* (62) found that of two lines of the Mahoney strain, one gave rise to the toxin and one did not. It may prove to be mere coincidence, but it is interesting that the one that did was a large plaque-former and the one that did not was a small plaque-former.

THE EFFECT OF VIRUSES ON CELL DIVISION

It frequently has been noted that infection of cells in culture by cytoidal animal viruses seems to cause inhibition of further cell division (8, 12, 27). In the usual culture system, however, this effect is not easy to measure, and it is difficult to separate from other degenerative effects on the cell. The question of whether or not animal viruses have a damaging effect on cell division apart from other cell-destructive effects is an interesting one, particularly in view of the well-known capacity of bacterial viruses to bring about cessation of cell division very soon after attaching to the bacterial wall (70). Newer cell culture techniques have recently allowed quantitative study of this problem with animal viruses.

The most extensive work has been that of Marcus & Puck (71 to 74), employing single-cell plating techniques. In these studies suspended cells were exposed to virus, washed, and then distributed in petri dishes as single cells in a medium inhibitory to any virus subsequently released from the cells. Cells capable of proceeding through continued division were later counted as colonies. Using Newcastle disease virus as the test virus, these workers have shown that attachment of only one virus particle is sufficient to prevent even one division of HeLa cells. This was found to be true whether the strain was a virulent one or a relatively avirulent one for the normal chicken host. This effect could be prevented by treatment of the virus with specific antiserum, or by previous irradiation of the virus with ultraviolet light. This latter point contrasts with the findings made with bacteriophages, for ultraviolet irradiation leaves some phages still with the capacity to inhibit host cell division (75). An interesting phenomenon was found with some lines of

HeLa cells, where increasing the multiplicity of infection above 4 resulted in a cell-sparing effect in which the cells survived, continued to divide, and produced colonies. There was indication that this was due to failure of virus penetration (72), and since reaction temperatures of about 20°C. and virus strains of high elution enzyme activity tended to increase the cell-sparing effect, Marcus (74) suggests that it is related to virus elution before the reaction responsible for inhibition of division is completed.

In the experiments of Marcus & Puck, adsorption of NDV particles to cells did not prevent cellular attachment to glass, spreading on the glass, or survival for periods of at least 20 hr. (72). The cells did not divide, however, and degenerated during the next 20 hr. In many of these cells, undoubtedly, the usual multiplication cycle of NDV proceeded and eventually was responsible for the destruction of the cell. An important point, however, was the demonstration that under the conditions of their experiments the number of cell-killing virus particles (cell-killing in the sense that the host cell can no longer divide) closely approximated the number of hemagglutinating particles, and often considerably exceeded the number of particles capable of initiating infection in eggs or of initiating plaque-formation in chick embryo cell monolayers (73). The relationship between cell-killing particles and plaque-forming particles varied with the NDV strain. The two activities were usually approximately equal for one strain, but for another the cell-killing particles regularly exceeded the plaque-forming units by as much as 10%. This clearly indicates that an NDV virus particle incapable of initiating an infection that results in release of infectious particles may still be capable of altering a HeLa cell sufficiently to prevent further division, and can thus effectively destroy the cell. Whether particles that are cell-killing but not plaque-forming accomplish their effect by some reaction short of replication of virus material, or whether they initiate an incomplete cycle akin to those of the myxoviruses discussed in a preceding section, has not yet been determined. The latter seems to be a possibility, however.

Stoker & Newton (76, 77) studied the influence of herpes virus on the division of HeLa cells isolated in microdrops, and also on HeLa cells undergoing waves of nearly synchronous division [parasynchronous division (78)]. With HeLa cells in microdrops they arrived at the conclusion that one HeLa plaque-forming unit is sufficient to prevent further division of the cell. In their viral inoculum, however, there were about 10 virus particles capable of initiating infection on the chick embryo chorioallantoic membrane for every one capable of producing plaques on HeLa cell monolayers. It could be demonstrated that the chick cell-infective particles adsorbed to HeLa cells, but they seemed not to be efficient in damaging mitosis in HeLa cells. This is in considerable contrast to the findings of Marcus & Puck with Newcastle disease virus, where the virus was found to be more likely to have the capacity for inhibiting HeLa cell mitosis than for initiating infection in chick embryo cells. The experiments on NDV and those on herpes virus were quite different in technique, and therein may lie the explanation of the contrasting

results. But it is conceivable, also, that the finding represents an important difference between these two viruses, or between RNA and DNA viruses, in their processes of approaching and modifying intracellular events.

Stoker & Newton initiated waves of cell division in HeLa cells by chilling them at 4°C. for 1 hr. and then returning them to 37°C. In these cultures 60 to 80 per cent of the cells divided within a period of 1 to 2 hr. beginning about 18 hr. after return to 37°C. By introducing herpes virus at various intervals before the expected mitosis, it was possible to demonstrate that at a multiplicity of 1.0 PFU/cell herpes virus could within 1 hr. establish whatever relationship is necessary for inhibition of cell division. Since their strain of herpes virus had a lag phase of 9 to 12 hr. in HeLa cells, this implies that the damage to mitosis is accomplished relatively early in the reproductive cycle of the virus.

The "cell-killing" effect of encephalomyocarditis virus described by Hoskins *et al.* (79 to 81) may be mentioned at this point. Not much detail is available as to its basis, but it has a few superficial similarities to the effects of Newcastle disease virus on HeLa cells. Hoskins *et al.* (79) and Sanders *et al.* (81) find in preparations of encephalomyocarditis virus a large number of virus particles that appear able to kill Krebs 2 carcinoma cells in suspension, but are not able to initiate plaque-formation on monolayers. Cell-killing is recognized by a change from the state in which the cells do not stain with eosin to one in which they do stain. Various manipulations of a virus preparation, such as heating or freezing, can result in a population of virus particles in which the cell-killing titer is 10 to 100 times higher than the plaque-forming titer. Cell-killing, as used by these investigators, has no special relationship to cell division, but the cells appear to lose viability and to take up eosin about 10 hr. after exposure to the virus. This is the time that the cells would begin releasing new infectious virus if infected with a plaque-forming particle. Those cells to which cell-killing particles adsorb do not release infectious particles, but examination of the cells for production of intracellular non-infectious viral components has not yet been reported.

Although general observation has suggested that many viruses have an inhibitory or damaging effect on cell division, it is to be assumed that further study will reveal many patterns and degrees of effect. The data on Newcastle disease virus and herpes virus represent currently available information about two viruses with severely destructive effects on mitosis. Even within herpes virus and Newcastle disease virus strains, examples of less severe effects can be pointed out. In most of these examples, however, detailed information is not yet available on the relationships between virus and cell and on whether the differences seen are due to strain differences or to circumstances of the experiments. Gray *et al.* (82), and Scott & McLeod (83) have described an effect of herpes virus on HeLa cells that results in a period of proliferation prior to cell degeneration. Wheelock & Tamm (84) have demonstrated with fluorescent antibody that after infection of HeLa cells with Newcastle disease virus or with influenza virus, cells containing viral antigen

appear to enter mitosis and to divide. Puck & Cieciura (85) have studied a HeLa cell-NDV carrier state in which virus is carried through division and clonal isolation of cells, but the location of the virus in or on these cells has not yet been described. Walker & Hinze (86) have studied human conjunctiva cell cultures chronically infected with mumps virus. In these cultures more than 90 per cent of cells can be shown to have viral antigen in their cytoplasm, yet their cloning efficiency is regularly 50 to 100 per cent. Cells in mitosis can be shown to adsorb erythrocytes to their surface and to have antigen in their cytoplasm, suggesting that they may be excreting virus either immediately before or during mitosis. Chick fibroblasts infected with Rous sarcoma virus have been shown to undergo mitosis (87), and it is to be expected that further *in vitro* demonstrations of division of cells infected with tumor viruses will appear.

THE RELATIONSHIP OF CELL DEGENERATION TO VIRAL-INDUCED METABOLIC ALTERATIONS

From the studies summarized in previous sections it seems clear that cellular damage, as evidenced by cytopathic changes in the cells, has no dependence on production or release of infectious virus particles. It is difficult to rule out completely the possibility that infectious particles add something to cellular damage, but it seems unlikely that this contribution is anything special or more than such particles would add as non-infectious units. In this respect it appears that animal viruses are consistent with bacteriophages, since the phage-infected bacterial cell may undergo lysis even though the contained virus has not achieved the status of infectiousness (35 to 37). In regard to the animal cell, however, is virus-induced degeneration independent of production of abundant quantities of virus materials? Admittedly, in this review the terms "virus components" and "virus materials" have been used rather loosely for, although the terms seem to imply that such materials represent precursors or subunits of the ultimate mature virus particle, it is often difficult to establish such a role or even to relate the materials to antigens within the mature particle by serologic means. In the instances of the materials produced in the abortive cycle of myxoviruses, and in the proflavine-inhibited poliovirus or foot-and-mouth disease virus infections, there has been little difficulty in demonstrating that the products are closely related to mature virus particles or to elements included in complete particles. The status of some materials found associated with other viruses may be less clear. Regardless of whether or not a virus-associated material can be considered a subunit, however, the production by a virus-attacked cell of large quantities of products that are like those regularly associated with infectious virus synthesis, and absent from normal cells, implies that cellular synthetic processes have been set in motion that are probably not different in important ways (as far as the cell is concerned) from those occurring in the ordinary complete, infectious cycle.

Of the examples that can be found of *in vitro* cell-damaging virus-cell

associations, most have been demonstrated to involve abundant synthesis of products, either infectious or non-infectious, characteristic of the virus replication process. Of the others for which there is sufficient information to provide any clues at all, there are reasons to suspect the involvement of an abortive or incomplete cycle. Further analysis may reveal that the fluorophenylalanine-inhibited poliovirus infection studied by Ackermann (10), the cell-killing reaction of Newcastle disease virus non-plaque-forming particles studied by Marcus & Puck (72), the cell-killing reaction of encephalomyocarditis virus studied by Sanders *et al.* (81), or the rapid effect of mumps virus on HeLa cells (47, 48) do not include such a multiplication cycle, but at this time there does not seem to be sufficient reason for concluding that these instances involve basically different processes. If there exists for animal viruses a reaction comparable to that seen with some virulent phages in which mere attachment of a virus particle to the cell surface results in cell death (75), the approach currently being employed by Marcus and Puck may reveal it.

All of this is quite apart from the effect of extraviral toxin-like materials such as those demonstrated in poliovirus and adenovirus infected cultures. There is not yet enough information about the effect of these factors on the cell to provide many hints as to mechanism of action, but the extreme rapidity with which the poliovirus-associated factor affects cells, and its apparent antigenic relationship to the cell rather than to the virus, suggest that it and its effects may be outside the pale of the more familiar virus-cell reactions.

If the major cell damaging relationships between virus and cell involve the synthesis of viral materials, this still does not tell us whether it is the process of multiplication or the products of the process that lead to damage of the cell. Only a few clues are available. The non-infectious viral materials produced in the abortive cycle of the myxoviruses (40, 42) or in the proflavine-inhibited poliomyelitis infected cell (38) seem not, in themselves, to be damaging to other cells. There is some suggestion from Pereira's study (27) of the extraviral toxin associated with the adenoviruses that this material may appear in significant quantities only relatively late in the multiplication process and, if it has any effect at all on the cell in which it is produced, the effect may perhaps be only that of altering membrane permeability to allow final release of virus particles. Examination of the effects of the extraviral toxin-like materials on infected cells and their contents at various stages of the multiplication cycle, as well as on otherwise normal cells, should help clarify their role. Unless some of the cell-damaging virus-cell relationships, such as the fluorophenylalanine-inhibited poliovirus infection, can be shown not to involve an abortive cycle, there does not yet seem to be a conclusive way of ruling out a damaging role for the products of virus synthesis. In spite of this, however, to most investigators the biosynthetic alterations required of the cell for production of viral components have seemed a much more reasonable explanation of virus-induced cellular damage.

Recent years have seen the beginnings of studies aimed at determining what metabolic changes are brought about in cells by viral infection, and how synthetic processes are altered to result in viral synthesis. The general approach has been one of comparing infected with uninfected cells in regard to changes in RNA, DNA, and protein, and the use of radioactive elements to follow the synthesis and breakdown of nucleic acids. Much of the attention, so far, has been focused on poliovirus infection, and only with this virus is there sufficient information to provide some outline of how biochemical events are related to the time sequence of virus production and cytopathic effect already discussed. The largest number and most detailed of studies so far reported, have been those of Ackermann and co-workers. Their work, although recently reviewed elsewhere (17, 88), will be outlined briefly here.

Attention has been concentrated on the first 7 hr. after infection of HeLa cells with type 1 poliovirus, since the important changes would be expected in this early period of the multiplication process. By 1 hr. after infection there is an increase in the incorporation of P^{32} into nuclear DNA, nuclear RNA, and cytoplasmic RNA (88 to 90). Incorporation into nuclear RNA continues until about the 4th hr. and then falls off abruptly. It is at about this time that the first infectious virus appears in the cytoplasm. Incorporation of P^{32} into nuclear DNA declines after about the 2nd hr. Incorporation into cytoplasmic RNA, however, continues at an increasing rate until about the 6th hr. at which time the rate of incorporation is two to three times greater than that in uninfected cells. There is then a decline in rate just before virus release begins. Ackermann and co-workers believe this increased incorporation of P^{32} reflects net synthesis of cytoplasmic RNA since they can show large increases in the RNA content of the cytoplasm of infected cells (90).

Within 1 hr. after infection, a detectable increase in cytoplasmic RNA begins and is accompanied by an increase in protein (90). Accumulation of cytoplasmic RNA continues at a constant rate until the 4th hr. when it increases still more, and then abruptly halts at about the 6th hr. At that time the cytoplasmic RNA level is about twice that of uninfected cells. Increased protein production continues at a constant rate until the 7th hr. when it is double that of control cells. Nuclear DNA and RNA show no increased accumulation over the uninfected cell. Ackermann *et al.* (90) point out that 50 per cent of the increased quantity of protein and RNA is formed before infectious virus makes its first appearance, and 50 per cent after the accumulation of virus begins in the cytoplasm. They emphasize, in addition, that the increases in protein and RNA are much greater than can be accounted for as virus particles.

Certain parts of the sequence of events outlined by Ackermann and his co-workers have been under study by other investigators, and there has been agreement and disagreement. Becker *et al.* (91) found an increase in incorporation of P^{32} into human amnion cells infected with type 2 poliovirus, but did not localize the increase. Miroff *et al.* (92) found increased uptake of P^{32}

into the total nucleic acids of infected HeLa cells. Goldfine *et al.* (93) found the incorporation of labeled cytidine into RNA and DNA inhibited for the first 5½ hr. after HeLa cells were infected with type 3 poliovirus. Rothstein & Manson (94) did not find increased incorporation of P^{32} or increased quantities of RNA in HeLa cells infected with type 2 poliovirus. Salzman & Lockart (95) did not find an increased turnover of RNA or DNA in infected cells and reported an inhibition of net RNA and protein synthesis. There has been considerable variation among investigators in the design of their experiments on poliovirus infected cells. One particular point that may be contributing to differences in results is that some workers, including Ackermann and his associates, are using cells held in a medium that does not allow continued growth and division of cells, while others are using cultures in continued growth.

Biochemical studies on cells infected with viruses other than poliovirus have not progressed as far. Work on cells infected with the adenoviruses points to increased incorporation of P^{32} into both DNA and RNA (96, 97) and to increased accumulation of DNA and protein in infected cells (98) during the course of the infection, but there have not yet been studies to relate biochemical changes to the time sequence of virus production or cell damage. The DNA changes are of interest here because these appear to be DNA-containing viruses. In HeLa cells infected with herpes virus, increased quantities of DNA appear in the nucleus during the period of 6 to 9 hr. after infection (99). This is before the intracellular appearance of infectious virus which is first detectable at about 12 hr. By 72 hr. after infection the nuclear DNA is increased to double that of uninfected cells. The related virus of pseudorabies seems also to cause increased synthesis of DNA in the nucleus toward the end of the latent period and throughout the period of virus increase (100). Again, the effect on DNA by these two viruses is of interest because of the likelihood that these are DNA viruses.

The biochemical alterations so far demonstrated in infected cells do not in themselves point to any special destructive process. It is noteworthy, furthermore, that not all studies of virus-infected cells have revealed significant biochemical alterations (94, 95, 101, 102), even though concerned with viruses that multiply vigorously, and some question remains as to what is fact and what is artifact. Among those investigators who have found marked alterations in cellular nucleic acids and proteins, however, almost all have been impressed by the fact that the observed increases in nucleic acids and proteins are considerably beyond what can be accounted for in the virus yield from the cell (17, 90, 98 to 100). This has reinforced the hypothesis (17, 103 to 105) that the action of a virus on a cell is primarily a stimulatory one, and that cell degeneration is secondary to a metabolic imbalance that diverts critical materials largely into some non-viral form of nucleic acid and protein where they are not available to meet the needs of the normal turnover of the cell. This fits well with the conclusion of many that the destructive effect of an animal virus cannot be accounted for by the relatively small

amount of nucleic acid and protein incorporated into virus materials. To the cell, however, it probably makes little difference whether the diversion is into virus components or into some other form of nucleic acid and protein; the important thing probably is the rate of the diversion, and upon this the survival of the cell may hinge. As data accumulate it may become possible to determine what rate of diversion a cell can tolerate, whether the diversion be into virus or into some other form. It might be expected that different patterns will be exposed for different viruses, with greater or lesser quantities of essential materials going to either end product, but with cell destruction as the same end result if a critical rate of diversion is exceeded.

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THE GENETICS AND CYTOLOGY OF CHLAMYDOMONAS¹

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INTRODUCTION

Haploid, unicellular green algae of the genus *Chlamydomonas* provide model systems for the investigation of an array of physiological and genetic problems and, for such investigations, they possess a combination of characteristics which make them particularly favorable organisms for research. Among the advantages for genetic analysis are a short life cycle, a classical meiosis, the production of tetrads which can be isolated rapidly, and a growth habit amenable to many of the techniques of bacterial genetics. It is feasible therefore, to undertake studies of gene recombination and to deal with sufficiently large numbers of progeny to permit high genetic resolution. The unicellular vegetative and zygote stages make it possible to investigate the fine structure of such cell organelles as the nucleus, chloroplast, pyrenoid, and eye spot. For several different species of *Chlamydomonas*, mutant strains are available which are of a morphological or physiological nature. Of special significance are those mutant strains which permit the investigation of such fundamental problems as the genetic control of pigment synthesis, of the primary steps in photosynthesis, and of biochemical pathways in a photosynthetic organism. Investigations of this type are possible because vegetative growth yields large numbers of cells in a relatively short period of time. Thus, genetic investigations can be coupled with biochemical studies and observations of cellular fine structure.

Until 1950, the only genetic investigations were those of Moewus (35). Unfortunately, the data from these investigations cannot be considered reliable because of the numerous unsuccessful efforts to reproduce them. Since that time, however, there has been amplified research activity with *Chlamydomonas*. This article will review the most pertinent studies of the past few years.

Interest in *Chlamydomonas* as an organism for research dates back to the initial cytological observations of haploid chromosome numbers by Dangeard in 1899 (3) and the early genetic studies of Pascher in 1916 and 1918 (39, 40) in which tetrad analysis was carried out for the first time in any organism.

MORPHOLOGY

Three species of *Chlamydomonas*, *C. eugametos*, *C. moewusii*, and *C.*

¹ The survey of the literature pertaining to this review was concluded in January, 1960.

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reinhardi are the principal ones that have been used in both genetic and cytological studies.

In gross morphology the vegetative cells of the species of *Chlamydomonas* considered in this review are ovoid in shape, being about $8 \times 15 \mu$ in their largest dimensions. The cells are motile when grown in liquid medium and swim with the aid of a pair of flagella which define the anterior end of the cell. On solid medium the cells possess or lack flagella and are nonmotile. When placed in liquid these cells become motile rapidly. The flagella may vary in length from 5 to 30μ . Vegetative cells contain a single chloroplast in which a pyrenoid and eyespot are imbedded. The nucleus is located outside the chloroplast. The details of the ultrastructure of the vegetative cells of *Chlamydomonas* will be considered in a later section of this review.

Mature zygotes of *Chlamydomonas* are spherical and are about 18 to 25μ in diameter. The wall becomes thickened as zygotes mature, and the ultimate thickness of the wall depends in part upon the intensity and duration of the light given during zygote maturation. This wall is characterized by having a coarse, reticulate structure, and it has been reported for *C. moewusii* (22) that it is birefringent and cellulose in nature. In addition, it has been shown (22) that during fusion the gametic wall is shed and the zygote becomes surrounded by a primary zygote membrane. The formation of this membrane occurs before the fusion of gamete nuclei. Upon illumination the true zygote wall begins to form, the zygote enlarges, and the primary zygote membrane is cast off. There have been no published studies of the fine structure of either the immature or mature zygote.

CONDITIONS FOR GROWTH

The medium for the growth of wild-type *Chlamydomonas* in the light consists of a few inorganic salts including certain trace elements. This medium can be modified by the addition of various nutritional supplements for the growth of auxotrophs (19, 23, 24, 46). Vegetative cell multiplication in the light is usually accomplished with the aid of daylight fluorescent lamps at a range of 250 to 800 ft. c. Rapid vegetative growth of *C. reinhardi*, with a generation time of 5 hr. can be achieved in a liquid medium in which there is a tenfold increase in the mineral salts, aeration with five per cent carbon dioxide in air (or air alone), and agitation in large cultures of a liter or more. Under these conditions a final cell concentration of 2×10^7 per ml. can be obtained.

Multiplication in the dark at least in *C. reinhardi* (50), *C. dysosmos*, and *C. debaryana* (25) can be achieved with the addition of sodium acetate to the minimal medium. *C. moewusii* has been reported to be an obligate photoautotroph (21) on the basis of attempts to obtain cell multiplication on 17 different organic acids, 7 alcohols, 4 hexoses, 6 pentoses, 6 disaccharides, 2 trisaccharides, 6 phosphorylated compounds including glucose-1-phosphate and glycerophosphate, and 12 miscellaneous compounds including soluble starch, peptone, tryptone, and yeast extract.

1950
Levine

Zygotes are matured on a minimal medium or, if necessary, on supplemented medium. In the case of *C. reinhardtii*, maturation on minimal medium is satisfactory for most crosses. However, in crosses wherein certain auxotrophic mutants are selfed, better germination is obtained on a suitably supplemented medium.

LIFE CYCLE

The life cycles of *C. eugametos*, *C. moewusii*, and *C. reinhardtii* are similar except for the fact that the gametes of *C. eugametos* and *C. moewusii* do not fuse immediately to form the zygote. Instead of fusing, a bridge is formed between mating pairs, and they remain in a tandem fashion for several hours before fusion is completed. The life cycle of *C. reinhardtii* will be described here (Fig. 1). When agar-grown, haploid, vegetative cells are suspended in water and illuminated, each cell becomes motile through the action of two flagella. After 2 to 4 hr. in the light, the motile vegetative cells differentiate into gametes by depleting their available nitrogen supply (45, 46). When gametes of opposite mating type are mixed, pairs or clumps containing cells of both

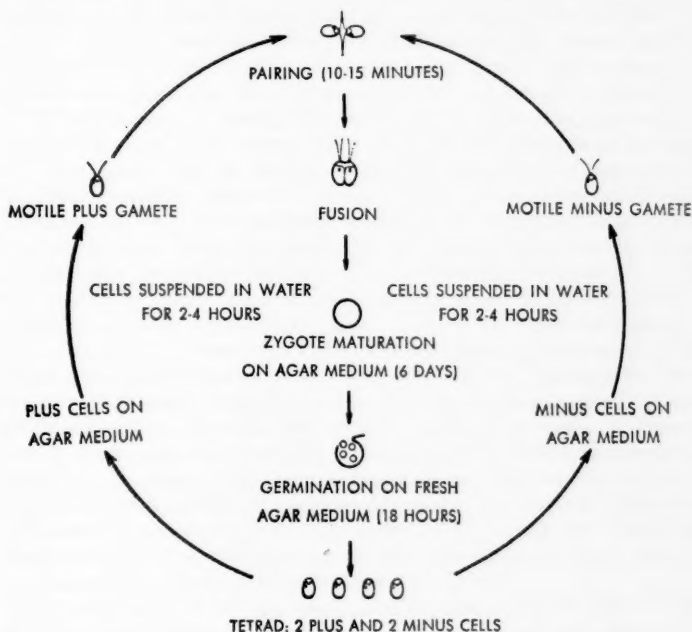


FIG. 1. The life cycle of *C. reinhardtii* [after Levine & Ebersold (18)].
Explanation in text.

mating type form immediately. Shortly thereafter, cells of opposite mating type begin to fuse in pairs. Ten to 15 min. after pairing begins, the process of cell fusion is completed by the formation of a binuclear, quadriflagellate zygote. At this time zygotes can be plated onto an agar medium where all mating ceases, and only those cells which have started to fuse will form mature zygotes. After the resorption or loss of their flagella, the zygotes develop a thick wall and enlarge to approximately twice their original diameter. The zygotes require a maturation period which, at 25°C., is six days in length. The first 18 to 24 hr. of this period is in the light (500 ft. c.) while the remaining time is in the dark. Following the maturation period, zygotes can be induced to germinate by transferring them to fresh agar medium and placing them in the light. The time required for germination varies from 15 to 24 hr., depending in part on the genotype of the zygote. At germination the zygote wall ruptures and four or, more frequently, eight haploid cells are extruded. These cells are the products of meiosis and are capable of vegetative growth.

ULTRASTRUCTURE

Sager (44) and Sager & Palade (47, 48) have studied the ultrastructure of *C. reinhardtii*, placing particular emphasis on chloroplast structure in wild-type and certain mutant strains. *C. reinhardtii* exhibits many of the cytological structures of the cells of higher animals and plants. In a series of excellent electromicrographs, Sager & Palade have shown the presence of dictyosomes, mitochondria, a double cell membrane, endoplasmic reticulum, and a fenestrated nuclear membrane. They have also shown that there are, in the vicinity of the dictyosomes, large, irregular vesicles or dilated cisternae. They suggest that connections exist between the chloroplast membrane and other membranous systems in the cytoplasm and "may be considered as the circulatory system of the cytoplasm" (48). However, much experimental work will have to be done before such an interpretation can be fully substantiated.

The structure of the chloroplasts of a number of lower and higher plants has been the subject of study by electronmicroscopy. It is clear that the chloroplasts of all organisms so far studied have in common the feature of an array of regularly packed double membranes or lamellae of high electron density. These membranes are often paired in higher plants and stacked into discs which represent the grana as seen with the light microscope. The double membranes of most algal chloroplasts, however, are not grouped into distinct grana but are continuous across the chloroplast. In *C. reinhardtii* the lamellae are double, but Sager & Palade (48) suggest that, unlike other algae, they are stacked into discs which in turn are surrounded by a double membrane. As such, these discs might resemble the grana of higher plants. Accordingly, it might be assumed that the *Chlamydomonas* chloroplast is an evolutionary transition between the chloroplasts of most algae and those of higher plants. Because there has been only a limited survey of chloroplast structure in the algae (28, 65), however, it is indeed premature to speculate on the evolutionary position that *Chlamydomonas* holds with regard to the possible

transition from the nongrana-containing chloroplast to the grana-containing chloroplast.

The laminate structure of the chloroplast is one of its most striking features. The functional significance of this laminate structure is not fully understood though it is possible to suggest that the lamellae are associated with the formation or presence of chlorophyll. Sager (44) and Sager & Palade (47) have examined the chloroplast structure in two mutant strains of *C. reinhardtii* as compared with the wild-type strain. Wild-type *C. reinhardtii* forms chlorophyll when grown in the light or in the dark, while the mutant known as *yellow* forms very little or no chlorophyll in the dark. It cannot be distinguished from wild-type when grown in the light. A second mutant, *pale-green*, can grow only in the dark since the effect of light is lethal. When grown in the dark, the *yellow* mutant shows the presence of a chloroplast as evidenced by the presence of starch grains surrounded by the outer chloroplast membrane. There is, however, almost a total absence of double-membraned lamellae. When grown in the light, the chloroplast structure of the *yellow* mutant is indistinguishable from that of the wild-type strain. Thus, the mutant phenotype is both the loss of ability to synthesize chlorophyll in the dark and to form a laminate system within the chloroplast. It has been reported (44) that chlorophyll synthesis in the light by the *yellow* mutant precedes the formation of lamellae by 7 hr. By this time 10 per cent of the final amount of chlorophyll has been formed.

The *pale-green* mutant also has a chloroplast and, like the *yellow* mutant, lacks an elaborate laminate structure. It does contain, however, a few lamellae which appear to be associated with the chloroplast membrane and about five per cent of the amount of chlorophyll found in the wild-type strain.

The correlation between chlorophyll and the laminate structure of the chloroplast in the *yellow* mutant suggests to Sager (44) that the synthesis of chlorophyll is essential to the formation of the lamellae. However, it is usual to sample only a few cells for electron microscopy, and from this sample the absence of lamellae in the entire cell population is inferred. The determination of chlorophyll, however, is based upon a relatively large sample from the same population of cells. If a cell population is heterogeneous with, in the case of the *yellow* mutant, 10 per cent of its cells having a complete complement of chlorophyll and fully formed chloroplasts, these cells may be missed in microscopic observations. Since the number of cells examined is not given, the argument above casts reasonable doubt on the validity of the hypothesis that chlorophyll synthesis precedes lamella formation or is necessary for it. Indeed, on the basis of present knowledge, lamella formation in the chloroplast of *C. reinhardtii* may either precede and be necessary for chlorophyll formation or be synchronous with it.

In addition to the chloroplast structure in *C. reinhardtii*, the fine structure of the flagella of *C. moewusii* has been investigated (9, 27). The flagella of *C. moewusii* are similar to other flagella and cilia, since they consist of a cylinder with nine double fibrils surrounding a center pair of fibrils. The fibrils appear

to be embedded in a matrix and are surrounded by an outer membrane which is approximately 100 A thick. Each fibril is 100 to 150 A in diameter. The flagella of the motile, wild-type strain and 11 different mutants with paralyzed flagella appear to be identical in structure.

CHROMOSOME NUMBERS

Buffaloe (1) has reviewed the early findings regarding chromosome numbers in *Chlamydomonas*. Several investigators have recently undertaken karyological studies of a number of species of *Chlamydomonas* (1, 20, 49, 63). Schaechter & DeLamater (49) report the following haploid chromosome numbers: *C. moewusii*, 36 ± 2 ; *C. eugametos*, 38 ± 4 ; *C. reinhardi*, 18 ± 2 ; and *C. dysosmos*, 16 ± 1 .

Wetherell & Krauss (63) report a haploid chromosome number of 16 for *C. moewusii*, *C. eugametos*, and *C. reinhardi*. On the other hand, Buffaloe (1) in a study of four species of *Chlamydomonas*, (*C. moewusii*, *C. eugametos*, *C. reinhardi*, and *C. chlamydogama*) has found a haploid chromosome number of eight for these species. A similar finding has been obtained by Levine & Folsome (20) for both mitosis and meiosis in *C. reinhardi*. In addition, as will be pointed out below, the haploid chromosome number of eight for *C. reinhardi* is in good agreement with genetic data for the number of linkage groups.

The discrepancy between the findings of Buffaloe and Levine & Folsome on the one hand and Schaechter & DeLamater on the other can be reconciled on a number of grounds. First, it has been shown (1) that a light intensity of 800 ft. c. induces temporary polyploidy in *C. eugametos* and *C. moewusii*. Schaechter & DeLamater do not indicate what light intensity they used. Second, early prophase stages of both mitosis and meiosis indicate the presence of a large number of chromatic bodies (1, 20) which later appear to condense to form eight chromosomes.

INDUCED VARIATION IN CHROMOSOME NUMBER

Wetherell & Krauss (63) have attempted to induce polyploidy in *C. eugametos* and in *C. reinhardi*. They report that after 55 hr. of exposure to a one per cent solution of colchicine, *C. eugametos* exhibits polyploidy. Unfortunately, the authors present no cytological evidence to substantiate the claim of polyploidy in this species, and it is doubtful, in view of the findings of Buffaloe (1), that they were able to determine accurately the haploid chromosome number. It would appear that the effects of colchicine on *C. reinhardi* were studied in somewhat more detail by Wetherell & Krauss. They report a haploid chromosome number of 16 for this species, which, as pointed out above, is not in agreement with the cytological observations of others (1, 20). Indeed, their photographs of normal haploid nuclei can be interpreted as showing either eight mitotic chromosomes which have undergone duplication or as early prophase nuclei in which the chromatic material has not yet condensed. The treatment of vegetative cells with a one per cent solution of

colchicine for 8 hr. was reported to cause a doubling of the chromosome number. A series of photographs of a single cell taken at different focal levels suggests that the number of chromosomes has increased following colchicine treatment. It is impossible, however, to determine the chromosome number from the depicted material. Other evidence is presented which is concerned with growth rates and dry weights of cells, and essentially identical results were obtained for both control and colchicine-treated cells of *C. reinhardi*.

More recently, Buffaloe (2) has shown that a 10 to 14 hr. exposure of *C. eugametos* to a 0.2 per cent solution of colchicine causes marked increases in cell diameter and nuclear mass. Nuclei of treated cells appear to be polyploid. When the colchicine-treated cells are transferred to a colchicine-free medium, the cells undergo a somatic reduction which restores the haploid chromosome number to the normal complement of eight.

Chromosome numbers in *Chlamydomonas* can be induced to vary with the light intensity to which vegetative cells are exposed. This has been shown for *C. eugametos* and *C. moewusii* (2). When these species are grown under 800 ft. c. instead of 100 ft. c. of light it was observed that the nuclear cycle was lengthened to 8 to 12 hr. in comparison with a cycle of 4 hr. observed at 100 ft. c. The cells were seen to enlarge, and the nuclei of these cells were ascertained to be polyploid. A series of what appeared to be endomitotic divisions then occurred until a number of haploid cells were released from within the old cell wall. The chromosome number of these cells was eight.

The mechanism whereby light induces alterations of the nuclear cycle is unknown. It can be postulated, however, that under conditions where light is not limiting, processes in the cytoplasm leading to cytokinesis become asynchronous with processes in the nucleus, particularly mitosis. The latter occurring independently of light intensity could then give rise to polyploid cells. It is interesting to point out that it was not possible to induce polyploidy with high light intensity in either *C. reinhardi* or *C. chlamydogama* (2).

THE NUCLEAR CYCLE

The nuclear cycle during meiosis in *C. reinhardi* has been followed from the time of mating until the end of the second meiotic division (20). This cycle can be divided into three parts. Part one consists of the initial stages of zygote formation beginning from the time of gametic pairing through gametic fusion to nuclear fusion. Fusion of gametes is very rapid, requiring about 5 min. The gametic nuclei fuse about 3.5 hr. later. This is to be contrasted with *C. moewusii* (26) in which cell fusion requires 1 to 2 hr. Nuclear fusion then occurs some 24 hr. later. Part two of the cycle in *C. reinhardi* is zygote maturation of which very little is known regarding the attendant cytological and biochemical alterations.

Part three of the nuclear cycle in *C. reinhardi* is meiosis of a classical sort in which the typical stages can be shown to occur. The entire meiotic process, which requires light, occurs in about 12 hr. with a series of recognizable stages occurring from the eighth to twelfth hour. Buffaloe (1) has described

a classical meiosis for *C. chlamydogama*, *C. eugametos*, *C. moewusii*, and *C. reinhardi*.

THE MITOTIC REPLICATION OF DEOXYRIBONUCLEIC ACID IN *CHLAMYDOMONAS REINHARDI*

Meselson & Stahl (30) have shown that the duplication of the DNA of *Escherichia coli* is semiconservative. That is, it occurs according to the model proposed by Watson & Crick (59). The technique of density gradient centrifugation (31) used in determining the pattern of DNA replication in *E. coli* has been employed in *C. reinhardi* by Sueoka (57) in order to determine the pattern of DNA replication in a classical chromosomal system. A series of experiments on mitosis in *C. reinhardi* has shown that DNA replication is semiconservative as is the case for *E. coli*. In addition, the experiments with *Chlamydomonas* are consistent with the results of radioautographic studies of chromosome duplication (58). Experiments on the meiotic replication of DNA are in progress.

METHODS OF GENETIC ANALYSIS

Two methods of genetic analysis are commonly used with *Chlamydomonas*. These are tetrad analysis and single-strand analysis from plating zygotes.

Tetrad analysis.—Various techniques have been developed for analyzing *Chlamydomonas* tetrads. All of the methods have in common the following steps: transferring zygotes, singly, to fresh agar medium, allowing time for germination, and separating the meiotic products.

The isolation of zygotes has been accomplished in a variety of ways. Smith & Regnery (51) and Lewin (24) cut out small blocks of agar, each with a single zygote, and arranged them quadrately or in rows on fresh agar medium. Sager (43) employed essentially the same method, but used a hypodermic needle to cut out a small cylinder of agar carrying a single zygote. The agar was then blown out by means of a hypodermic syringe. After germination a drop of water was placed on each zygote. The zygote products became motile and dissected themselves out of the zygote wall. When the water dried, each cell became immobilized on the agar surface where it was able to divide to form an isolated colony. This procedure has two main drawbacks. Since the time required for germination in some crosses varies from 14 to 24 hr., water must be added over a period of several hours. It is rather difficult to keep the drops from running together if they are to be large enough to assure good cell dispersal. Second, if only two or three colonies develop from a zygote it is very difficult to determine whether or not these are mixed or whether one or two products died.

Another method was developed (4, 5, 18) in which mature zygotes together with unmated vegetative cells are scraped from the agar surface with a small metal spatula and placed on fresh agar medium. Single zygotes are then manipulated, singly, into one end of 3 mm. \times 25 mm. rectangular lanes between parallel cuts in the agar surface (Fig. 2a). In order to kill the vegeta-

tive cells each plate is inverted for 35 sec. over a Petri dish containing chloroform. After germination (Fig. 2b), the zygote products are separated by means of a fine glass loop with the aid of a dissection microscope at 60 \times to form a row of cells 3 mm. apart within each lane (Fig. 2c). With a little experience several investigators have found this method practical, since it is possible to isolate the products from at least 100 zygotes in an hour. After colonies approximately 0.75 mm. in diameter develop, they can be replica-plated, using smooth-surfaced filter paper, to any combination of scoring media.

Zygote plating.—The zygote-plating method (18, 19) provides a means

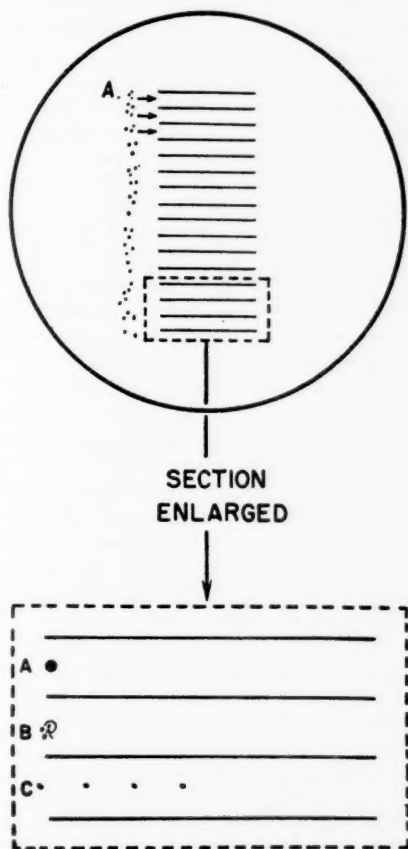


FIG. 2. Isolation of zygotes and zygote products for tetrad analysis in *C. reinhardtii* [after Levine & Ebersold (18)]. Explanation in text.

for analyzing a large number of single strands for recombinational events. Mature zygotes are suspended in distilled water and plated at concentrations of 100 to 2000 or more per plate. The products from each zygote are not separated but remain together in a single colony. By replica-plating to the proper media, the frequency of a desired genotype can be determined. An advantage of this technique is that it is possible to test for reciprocal recombination by analyzing the original colony from which the recombinant genotype was found.

MUTANT STRAINS OF *CHLAMYDOMONAS* AND THEIR GENETIC ANALYSIS

Mutant strains which differ from wild-type with respect to morphological, physiological, and nutritional characteristics have been obtained spontaneously and following ultraviolet and x-irradiation in *C. moewusii* (23, 37), *C. eugametos* (10, 37, 61, 62), and *C. reinhardi* (4, 7, 11, 12, 43, 60). The various kinds of mutants found and the available information concerning their genetic behavior is described separately for each of the three species.

C. moewusii.—Among the 194 mutant strains isolated by Lewin (23) the majority were *slow-growing* or *palmeloid*. Three nutritionally-deficient strains require, respectively, thiamine, *p*-aminobenzoic acid, and a source of organic carbon such as acetate for growth. The remaining 59 mutants were given general descriptions based upon motility, colony morphology, and pigmentation. Tetrad analysis indicated that several of the mutant strains such as *paralyzed flagella*, *non-photosynthetic*, *thiamine*, *p*-aminobenzoic acid, and *mating type* segregate in 2:2 fashion. Evidence for linkage was obtained for the markers *p*-aminobenzoic acid and *mating type*.

C. eugametos.—Several of the mutant strains recovered by Gowans (10) were similar to those of *C. moewusii* listed above. In addition, Gowans isolated strains described as *mottled colony*, *streptomycin-resistant*, *twirlers* (cells spin like a top instead of swimming in the normal fashion), and high *osmotic pressure* (growth occurs only on media having a higher osmotic pressure than the minimal medium used). Among 43 nutritionally-deficient mutant strains, requirements were found for nicotinamide, purines, carbon source, thiamine, and *p*-aminobenzoic acid. From the results of the analysis of over 800 tetrads it was shown that 13 of the nutritionally-deficient mutant strains represented single-gene mutations and that none of these were linked. Gene-centromere distances have been calculated for ten mutant genes and the mating type locus. Of considerable importance is the fact that no evidence was found from the analysis of 570 complete tetrads for the nonallelic genetic nature of the mating type loci as reported by Moewus (33).

From a study of x-ray-induced mutations in *C. eugametos*, Wetherell & Krauss (61, 62, 64) recovered a number of apparently unique mutant strains. These strains were found to have a nonspecific vitamin requirement since growth could be stimulated by choline, thiamine, *p*-aminobenzoic acid, and in some strains by nicotinic acid, pyridoxine, folic acid, and pantothenate. It was reported that in minimal medium all of these mutants attained a

growth rate equal to, or exceeding that, of wild-type after a lag period of from 1 to 20 days. From crosses of two of these mutant strains to wild-type, an unspecified number of tetrads yielded one normal:one mutant:two dead cells.

In an attempt to explain the behavior of these strains, the genetic nature of *C. eugametos* was investigated. Wetherell & Krauss interpreted the results of a comparison of x-ray survival curves of *C. eugametos* with those of haploid and diploid *C. reinhardi* (but see above: Induced Variations in Chromosomal Number) as indicating that *C. eugametos* possessed a high degree of genetic duplication. In addition, the fraction of subnormal-sized colonies surviving x-radiation could be associated with the degree of genetic duplication. It was postulated that the phenotype expressed by

many of these mutant strains was due to chromosomal deletions in segments of the genome which are normally duplicated. This is thought to create a condition of weakened synthetic capacity which is expressed as a pronounced lag in growth under the exacting conditions of culture in minimal medium.

The lag phenomenon is not substantiated by the data presented for growth of a typical mutant strain. When the graphs presented are replotted on a semilogarithmic scale, it is evident that the time required for cell division in minimal medium is more than twice that in the vitamin-supplemented medium and that the apparent lag in minimal does not exist. Unfortunately, the experiments were not designed to detect any appreciable growth in very young cultures.

If the colonies of subnormal size are, for the most part, due to multi-hit chromosomal aberrations as suggested by Wetherell & Krauss, approximately 32 per cent of all *C. eugametos* colonies surviving a 9000 r dose would represent such alterations. This should be amenable to genetic test which would reveal their nature in a more direct manner.

C. reinhardi.—The majority of the genetic investigations of *Chlamydomonas* have involved the use of the same strain of *C. reinhardi*. This strain was isolated originally by the late G. M. Smith. The work on *C. reinhardi* began with the demonstration by Smith & Regnery (51) that mating type was determined by a single pair of alleles.

Weaver (60) recovered several mutant strains following ultraviolet irradiation that were *pH-sensitive*, *sulfanilamide-resistant*, and *pigment-deficient*. One of these mutant strains, which was able to form protochlorophyll, but was unable to convert it to chlorophyll in darkness, was found to represent a single-gene mutation. Similar mutants have also been obtained by Sager (43) and Eversole (7).

Six mutant strains isolated by Sager (43), either spontaneously or following ultraviolet irradiation, were found to represent single-gene mutations. Gene-centromere distances were calculated for *streptomycin resistance*, *methionine sulfoximine resistance*, *yellow*, *yellow-green*, *pale-green*, *brown*, and *mating type*. None of these markers were found to be linked to one another.

Hartshorne (12) isolated a mutant colony following ultraviolet irradiation in which the eye spot was lacking in individual cells. Analysis of crosses of this mutant to wild-type indicated that the presence or absence of an eye spot (*ey*) was due to a single pair of alleles. The crosses also revealed the presence of two genes affecting colony size. According to Hartshorne's interpretation, the genes for small colonies (*sc*) and slow growth (*sg*) are present in the wild-type strain but are suppressed by the wild-type allele of *ey*. Thus, the *sc* and *sg* phenotypes can be expressed only when present with the mutant allele of *ey*. Since the *ey* strain possessed a normal colony size, mutation of *sc* and *sg* to their wild-type alleles must have accompanied the mutation of *ey* to its mutant state. Although the data presented are in agreement with the above interpretation, the 2:2 segregation of *sc* and *sg* has not been demonstrated directly.

Following ultraviolet and x-irradiation, Nybom (37) recovered mutations affecting drug resistance, morphological characteristics, and nutritional requirements in *C. moewusii*, *C. eugametos*, and *C. reinhardi*. A few morphological mutants, presumably of *C. reinhardi*, have shown 2:2 segregation when crossed with wild-type.

Among 32 mutant strains recovered following ultraviolet irradiation by Ebersold (4), the majority were slow-growing on all media tested. Others were described as *arginine-dependent*, *flagella-less*, *pigment-deficient*, *non-heterotrophic*, *raised colony*, and *non-autotrophic*. From the genetic data obtained, five markers and the centromeres were located in two linkage groups.

Eversole (7) studied 26 stable mutant strains requiring single growth factors, that were obtained after ultraviolet irradiation. Included among these were strains which required acetate, *p*-aminobenzoic acid, nicotinamide, thiamine, and arginine. The single-gene nature of these mutations was demonstrated by genetic experiments and three cases of linkage were found.

More recently, the mapping of available markers has been continued by the authors. The majority of the nutritional mutant strains used in this study were those described by Eversole or were mutants possessing unknown requirements and made available by him. Lewin kindly isolated several paralyzed flagella mutants of *C. reinhardi*. At the present time 35 mutant genes and the centromeres have been mapped in nine linkage groups (Table I). Linkage groups I through V have been definitely established. More data are necessary to secure groups VI through IX as well as for the localization to linkage group of a number of additional mutant strains. A detailed study of linkage group I, using four mutant loci, was made by the analysis of 1721 tetrads (5). The data obtained indicate that chiasma interference is positive and that chromatid interference is absent in that portion of the chromosome studied.

THE TIME OF CROSSING OVER

One of the primary objectives of many of the genetic investigations of *Chlamydomonas* has been to determine the time of crossing over. The stimu-

TABLE I
THE LINKAGE GROUPS OF *C. reinhardtii**

I	<i>c</i>	<i>ac-14</i> . . . 2 . . . <i>ac-14</i> <i>arg-1</i>	<i>ac-14</i> <i>arg-2</i>	<i>pab-2</i>	<i>thi-3</i>
		12	6	15	30
II	<i>c</i>	<i>ac-60</i> <i>164c</i>	<i>pf-12</i>	<i>pf-18</i>	<i>nic-2,3</i>
		20	10	8	8
III	<i>pf-15A</i>	<i>c</i> . . . 6 . . . <i>c</i> <i>pab-1,3</i>		<i>thi-2</i>	
		21		30	
IV	<i>pf-20</i>	<i>thi-4,6,7</i>	<i>c</i>	<i>nic-11</i>	
		30	7	1	
V	<i>ac-31,4</i>	<i>thi-8</i>	<i>c</i>	<i>pf-1</i>	
		5	10	20	
VI	<i>c</i> . . . 23 . . . <i>c</i> <i>nic-7,8</i> <i>mt</i>			<i>pf-14</i>	
			36		
VII	<i>c</i> . . . 4 . . . <i>c</i> <i>pf-17</i>		<i>ac-1</i>		
			4		
VIII	<i>c</i> . . . 12 . . . <i>c</i> <i>thi-1</i>			<i>ac-157b</i>	
			19		
IX	<i>ac-51</i>	<i>c</i> . . . 17 . . . <i>c</i> <i>pf-16</i>		<i>pf-13</i>	
		19		37	

c—centromere
ac—acetate-requiring
arg—arginine-requiring
mt—mating type
nic—nicotinamide-requiring
pab—*p*-aminobenzoic acid-requiring
pf—paralyzed flagella
thi—thiamine-requiring

* As of January 1960.

lus for this work was provided by Moewus (34, 36) who reported that in *C. eugametos*, crossing over occurred at the 2-strand stage at temperatures below 5°C. but at higher temperatures crossing over occurred at the 4-strand stage. It has been shown that in *C. moewusii* (24), crossing over between linked loci occurs at 4-strand stage at 23°C. The same result was ob-

tained for *C. reinhardi* (4) when zygotes were germinated both at 5°C. and at 26°C. Gowans (10) conducted more extensive experiments using *C. eugametos* in which zygotes were matured at 5, 13, 20, 25, 28, and 30°C. Zygotes were placed at the same temperatures for germination. Although zygotes which received the 5° and the 30°C. treatments failed to germinate, the results obtained from zygotes placed at the remaining temperatures revealed no indication of crossing over at the 2-strand stage.

Eversole & Tatum (8) and Ebersold (4) have presented tetrad data from crosses involving linked loci in *C. reinhardi* in which nonparental ditype (NPD) tetrads (tetrads in which all four strands are recombinant) were in excess. As pointed out by Eversole & Tatum, tetrads of this type could result from occasional crossing over at the 2-strand stage or could be the result of negative chiasma interference or positive chromatid interference. In the latter two cases NPD tetrads would result from 4-strand double cross overs. An attempt was made by Ebersold & Levine (5) to determine the phenomenon responsible for the reported excess NPD tetrads. An analysis of 12,286 zygotes by the zygote plating method revealed no such excess, and from 1721 tetrads involving four linked markers, only two tetrads might have resulted from crossing over at the 2-strand stage. It was concluded that although crossing over at the 2-strand stage in *C. reinhardi* could not be ruled out, it is a no more likely occurrence than in any other organism in which NPD tetrads have been found.

EXPERIMENTAL STUDIES OF GENE RECOMBINATION

Effects on the frequency of gene recombination by temperature, ultraviolet irradiation, and various metallic ion deficiencies have been studied with *Chlamydomonas* in an effort to gain some insight into conditions which might lead to a further understanding of the mechanism of recombination.

There are several reports in the literature (14 to 17a, 52 to 55) which suggest that the cellular ionic environment may play a role in the stability of chromosomes. Its role in crossing over in *C. reinhardi* was studied by Eversole & Tatum (8) and subsequently by Levine & Ebersold (19). Using the linked markers *arginine-1* and *arginine-2* of *C. reinhardi*, Eversole & Tatum reported that when gametes were suspended for 25 hr. in a 10^{-4} M solution of the chelating agent, ethylenediamine tetracetic acid (EDTA), the frequency of recombination was increased from 6 per cent to 59 per cent. In addition, they showed by flame photometry that significant amounts of calcium and magnesium were removed from the treated gametes by the action of the chelating agent. In their experiments they analyzed crossing over in about 650 zygotes which had been derived from treated gametes. In the experiments of Levine & Ebersold, over 60,000 zygotes were analyzed, and no evidence for increased recombination was found. Furthermore, it was found that a calcium deficiency, obtained by raising vegetative cells for several generations on a calcium-free medium, had no effect on the fre-

quency of recombination. Finally, using the radioactive isotope, Ca^{45} , it was found that EDTA was no more effective than ion-free water in removing calcium from gametes. The question of the possible role of metallic ions in the stability or structure of chromosomes still remains open. However, there is no clear evidence for such a role in so far as crossing over in *C. reinhardi*.

Gene recombination in *C. reinhardi* can be altered by irradiation of gametes with ultraviolet light (18). When gametes of one mating type are irradiated the zygotes obtained upon mating to unirradiated gametes produce tetrads about 10 per cent of which have aberrant segregation ratios. That is to say, there are ratios for given markers of 3:1 or 4:0. This result is superficially similar to that obtained by Roman & Jacob (41) who found that ultraviolet irradiation increased the frequency of aberrant segregation during mitotic recombination in yeast by several orders of magnitude. It has been suggested that the ultraviolet irradiation produces lesions in a chromosome and thus impedes normal chromosome duplication (13). Duplication can, however, occur if the replication process is switched to the homologous chromosome (13). In *Neurospora* (32), yeast (29), *Aspergillus* (56), *Sordaria* (38), and *Chlamydomonas* (18) aberrant segregation ratios, particularly those which are 3:1 ratio, have been variously explained as gene conversion (29) or as some form of gene recombination by partial replication or copy choice. Some, though certainly not all such aberrant segregations may be due to polysomy or polyploidy rather than a special form of gene recombination (56). For example, if, at the time of mating in *Chlamydomonas*, three cells rather than two fuse to form a zygote, meiosis in this triploid could lead to an array of peculiar segregation ratios.

Recent results from this laboratory with *C. reinhardi* suggest that spontaneous and ultraviolet-induced aberrant segregations can arise from the formation of a zygote which results from the fusion of three gametes. The evidence which supports this idea comes from two kinds of experiments. First, when the products of aberrant tetrads from ultraviolet irradiation of one mating type were crossed to wild-type, segregation occurred for a variety of markers which had not been seen in the products but which were seen in the original parents. This segregation would suggest that the products of the aberrant tetrads were not haploid in nature, but disomic. The second kind of evidence comes from the fact that the triple fusions in *Chlamydomonas* do occur. In order to perform a genetic test for the occurrence of triple fusions a cross was made in which three different mutant strains were combined. Tetrads from this cross were analyzed and it was found that certain of them produced products which carried markers from all three parents. There can be only one explanation for the origin of such tetrads and that is through a triple fusion. Further analysis of these products, through test crosses, has shown that certain of them are heterozygous for markers found in the original parents. This segregation for other markers suggests again that the products of these tetrads which arose by fusion of three nuclei are not haploid.

CYTOPLASMIC INHERITANCE

Sexually reproducing unicellular organisms provide excellent material for the investigation of a variety of aspects of cytoplasmic inheritance as exemplified by the studies of Ephrussi and co-workers with yeast, and Sonneborn with *Paramecium* (6).

One instance of cytoplasmic inheritance has been reported for *Chlamydomonas* (42). The wild-type strain of *C. reinhardi* is sensitive to streptomycin, but two classes of streptomycin-resistant mutants have been found. The first class is inherited in a normal Mendelian fashion while the second class exhibits a peculiar form of unilateral inheritance. This latter class is the non-Mendelian or cytoplasmic mutation which has been studied by Sager.

The original non-Mendelian mutant was obtained in the *plus* mating type and when crossed with wild-type gave rise to tetrads all of whose products were streptomycin-resistant. These products, however, segregated 2:2 for other markers including a pigment mutant and the mating type locus.

When the mating type *plus* progeny of these tetrads were crossed with various sensitive strains, all of their progeny were resistant. On the other hand, with certain rare exceptions, the resistance was not transmitted by the mating type *minus* progeny.

By means of a series of genetic tests Sager concluded that the resistance to streptomycin was controlled by extra-chromosomal factors. The nature, mode of transmission, and action of these factors are as yet unknown. Sager suggests, however, that the unilateral mode of transmission may be based upon a nonchromosomal determinant for streptomycin resistance which is in some way associated with the *plus* mating type. The hypothesis requires that the two mating types make an unequal contribution of cytoplasm to the zygote and that this contribution is ordinarily made by the *plus* mating type. The exceptional cases which arise when transmission is via the *minus* parent might be due to an occasional cytoplasmic contribution by that parent. Cytological observations (20) suggest, however, that both parents contribute their cytoplasm to the zygote. However, cytological observations are not sufficient to rule out the possibility of an unequal contribution of cytoplasm. Another possibility suggested by Sager is one in which it is assumed that a gene in the *plus* mating type or the mating type locus itself controls the activity or production of a cytoplasmic determinant for streptomycin resistance. The exceptional cases could be attributed to a sufficient amount of the cytoplasmic determinant in the mating type *minus* parent to permit its transfer to a zygote where it would once again come under the control of the gene associated with the *plus* mating type.

The first alternative, that of an unequal contribution of cytoplasm by one or the other parent, could be tested by following the mode of transmission of certain cytoplasmic organelles, in particular the chloroplast. For example, there are a number of mutants which have chloroplasts lacking the normal structure (see "Ultrastructure" above). If it is possible to obtain a pigment mutant with an aberrant chloroplast which is inherited in a non-

Mendelian fashion, it could be ascertained which of the two parents contributes the abnormally constituted chloroplast to the zygote and in this way determine whether or not there is an unequal distribution of cytoplasm or of cytoplasmic constituents.

PERSPECTIVES FOR FURTHER RESEARCH

Most of the work described in this review is of a descriptive sort essential to any further work on either the genetics or cytology of *Chlamydomonas*. It is now possible, particularly in the case of *C. reinhardi*, to pursue certain of the lines of investigation alluded to in the introduction of the review. Accordingly, a few perspectives for further research are summarized below.

The ease of tetrad analysis and the zygote plating method in *Chlamydomonas* provide an opportunity for studies of gene recombination. The details of the linkage groups in *C. reinhardi* are becoming known and, at present, an effort is being made in this laboratory to map a variety of closely linked, functionally related loci. With this accomplished, it will be possible to analyze genetic fine structure in *Chlamydomonas*. Such an analysis must be accompanied by an investigation of a variety of biosynthetic pathways under genetic control in *Chlamydomonas*. The functional blocks represented by the acetate, arginine, nicotinamide, *p*-aminobenzoic acid, and thiamine-requiring mutant strains need to be investigated. This is particularly true in cases in which very close linkage has been observed among functionally related mutant strains.

Of special interest are the 30 mutant strains of *C. reinhardi* which cannot grow in the light unless the minimal medium is supplemented with a carbon source such as sodium acetate. Some of these mutant strains are apparently completely or very closely linked. Tests for the growth of the acetate-requiring strains have been carried out in this laboratory using a variety of carbon sources similar to those cited by Lewin (21). In the case of two mutant strains, pyruvate has been found to be effective, at least for growth in the light. No carbon source other than acetate has been found to support growth in the dark for either the wild-type or the mutant strains.

As is the case for the "acetate" flagellates, the acetate requirement for the mutant strains of *C. reinhardi* is poorly understood. However, recent experiments from this laboratory have shown that the photosynthetic apparatus of several of the mutant strains is intact. That is to say, they possess a Hill reaction, can fix carbon dioxide, and can carry out photophosphorylation. It appears, however, that for certain mutant strains the Q_2^{CHL} , as measured by the Hill reaction is markedly reduced in comparison to that of the wild-type strain. Additional information may be gained from a quantitative study of the pigments in each of the mutant strains as well as from an investigation of the products formed after carbon dioxide fixation.³

³ Recent investigations (17b, 17c) have demonstrated that one of the acetate-requiring mutants, though normal in its pigments and chloroplast structure, is incapable of photosynthetic growth. It has been shown that the mutant cannot carry out photosynthetic phosphorylation.

There are several mutant strains of *C. reinhardi* in which chlorophyll synthesis is affected. The *yellow* and *pale green* (44) are two such mutant strains. There is, in addition, a mutant strain, *arginine-1* (*arg-1*), under investigation in this laboratory, in which it has been found that both chlorophyll-*a* and -*b* are lost when the cells are cultured in limiting arginine. Such a loss of chlorophyll does not occur in the case of another arginine-requiring strain, *arg-2*. The *arg-1* strain will grow on ornithine, citrulline, and arginine (4), and thus the block precedes ornithine. The *arg-2* strain grows on arginine only and therefore the block lies between citrulline and arginine. The relationship between the arginine requirement of *arg-1* and the loss of chlorophyll is currently under investigation.

There are mutant strains of *C. reinhardi* and *C. moewusii* which possess paralyzed flagella, as well as suppressor mutations of these paralyzed strains. These mutant strains provide excellent material for an investigation of the genetic control of motility, and those of *C. moewusii* are being investigated by Lewin. Since it has been shown (9) that the flagella of the paralyzed mutants appear to be indistinguishable from those of the wild-type strain, an immunogenetic approach would seem to be feasible. The techniques of immunodiffusion and immunoelectrophoresis may reveal interesting antigenic differences among the different paralyzed mutants. The effects of suppressor mutants on the antigenic character of the paralyzed mutants can also be studied.

As mentioned earlier in this review, *Chlamydomonas* provides a combination of characteristics which make it a favorable organism for investigations in which it is possible to attack fundamental problems in the biology of a photosynthetic organism from the point of view of its genetics, biochemistry, and cytology. A few of these problems have been mentioned here, some of which are currently now under investigation. The establishment of basic techniques for the genetics and cytology of *Chlamydomonas* should, however, broaden the scope of the problems to be investigated and it is hoped that the present review will be of some help in this regard.

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FINE STRUCTURE OF VIRUS-INFECTED CELLS¹

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In rendering an account of current information on the fine structure of virus-infected cells, the authors feel obliged at the outset to indicate its extent and limitations. First, the article is in no sense comprehensive, but represents rather a selective and critical review of the more recent literature. Secondly, emphasis is placed primarily on observations made with the electron microscope, and especially on those in which ultrathin sections of infected tissue were examined. Lastly, although the chief purpose is to consider changes in cellular architecture that occur—or fail to occur—in association with viral infection, the fine structure of viruses themselves, as observed to be present or to develop in cells, is also discussed.

It is important to recognize that the electron microscopist encounters several difficulties in attempting to ascertain the effects of viral agents on host cells. One of these is occasional distortion of fine structure which may occur either during fixation and dehydration of tissue, or in the subsequent embedding-process, or both. The observable effects may include variable loss of characteristic cytoplasmic and nuclear components, irregular aggregation of nuclear substance, disruption of nuclear and mitochondrial membranes, disorganization of the lamellae that constitute the endoplasmic reticulum, and disarrangement of cytoplasmic granules (112). When such changes seem obviously to be artifacts, the specimen is usually discarded for one that appears to be better preserved. Distortion may vary, however, in different parts of the same block of tissue and even within individual cells, especially when it has been caused by irregular polymerization of the embedding plastic at multiple foci. Therefore, the presence of a well-preserved cell adjacent to one that shows abnormal features does not necessarily mean that the first is healthy and the other diseased. Other technical factors that may play a part in altering the appearance of cells are impact of the microtome knife and exposure to the electron beam (110).

Another problem involves the understanding of requirements for suitable controls. Far too frequently attempts have been made to determine the specific effects of a virus by comparing the appearance of infected and normal cells. Only by examining the consequences of infection with different viruses, as well as the changes that are seen in cells undergoing spontaneous degeneration, is it possible to recognize pathologic alterations which may be significantly related to the virus under study, or those which, in certain instances, may be pathognomonic. In this connection, it should be pointed out that the results of electron microscopic examination of ultrathin sections

¹ The survey of the literature pertaining to this review was concluded in January, 1960.

of virus-infected tissue can frequently be compared advantageously with those obtained by the methods of light microscopy, using contiguous thick sections of the same tissue (115). But it must also be stated that alterations in the so-called "fine structure" of infected cells, as revealed by the light microscope in thick sections after they have been stained by various techniques, may bear little evident relation to the changes seen at much higher resolution in the electron microscope. One of the chief reasons for this frequent and sometimes seemingly paradoxical lack of correlation is simply that the light microscope records a much more composite picture of cellular elements in depth, since the sections are generally 100 to 500 times thicker than those used for electron microscopy. An important consequence of this basic technical difference is that "inclusion bodies," which are well known to be associated with certain viral infections (60), often cannot be recognized as such in thin sections, although some of their components may be seen and even identified (45). Therefore, in describing changes in the structure of an infected cell at the electron microscopic level, the term "inclusion body" becomes incongruous, and attention is centered on the finer details of both cell and infecting agent (11).

Finally, it is important to recognize that the electron microscopist is confronted by what may be entitled "sampling error" in his attempt to visualize viruses, to study their developmental stages, and to relate them to cell structure. Only about 1/500 the volume of any cell is contained within a section. Moreover, high resolution micrographs are the sole means whereby the finer details of viral and cellular structure may be observed. Considering the size of the sample actually examined in comparison with the mass of cells in a tissue culture or in a fragment of whole tissue, it becomes obvious from statistical considerations that a certain degree of caution must be exercised in interpreting electron microscopic data. The problem becomes even more significant when the changes accompanying viral multiplication are confined to small loci, or do not occur regularly in every cell. Difficulty also arises when an attempt is made to correlate structure with function. Unlike most immunologic and chemical data, which usually represent composite results obtained from the interaction of large viral and cellular populations over an extended period of time, electron micrographs depict only a small portion of the cell and its contained virus at a single moment (116, 119).

VIRUSES DEVELOPING PRIMARILY IN THE CYTOPLASM

Certain viruses appear to develop primarily in the cytoplasm, as judged by the criteria of morphology. There is evidence, however, that the nucleus participates in many, if not all, instances in the production of substances which either represent actual components of the virus or contribute to the mechanism of viral synthesis.

Pox viruses.—This group of agents includes a number of viruses which resemble each other morphologically and, so far as is known, undergo a similar type of developmental cycle in infected cells. Nevertheless, they are

capable of producing quite different patterns of disease in animal or human hosts, ranging from those that are characterized by a cellular response (pock formation) from which the group derives its name, e.g., vaccinia, fowl pox, ectromelia, etc., to others that are associated with a proliferative or neoplastic kind of reaction, e.g., molluscum contagiosum and Shope fibroma. It has, of course, been known for many years that infection by some members of this group is regularly accompanied by the appearance in cells of juxtanuclear cytoplasmic inclusion bodies which are readily visible in the light microscope and possess eosinophilic properties (60). These inclusions are also intensely Feulgen-positive (128). Electron microscopic examination in some instances has also revealed corresponding cytoplasmic aggregates of mature viral particles, apparently embedded in some kind of matrix which may be strongly osmophilic, suggesting the presence of increased amounts of lipids (45, 95, 124). However, it must be emphasized that such aggregates often are difficult to find, and that their occurrence probably depends largely on differences among strains of virus, the origin of infected cells, and the stage of infection when cells or tissues are collected for study.

Early in the course of infection with representative members of this group (vaccinia and fowl pox virus), cells often show localized areas of the cytoplasm in which the normal components are replaced or displaced by collections of finely granular material. Membranes appear to form at the periphery of such areas and progressively to enclose the granular material, which will constitute the so-called viroplasm (105, 158). At other foci, either within or adjacent to these finely granular regions, very dense material appears and breaks down into smaller units which will form the inner bodies or "nucleoids" of the viral particles (105). At this stage the virus usually appears to be oval—the elliptical shape probably reflecting compression of the section by the impact of the microtome knife—and has major and minor axes approximately 300 m μ and 180 m μ , respectively (96, 105). Individual particles typically have an outer limiting membrane, a granular viroplasm, and an inner, dense, oval nucleoid, which is usually placed eccentrically and measures about 60 \times 120 m μ . Single sections of optimal thickness (30 to 40 m μ) exhibit numerous particles which either fail completely to show nucleoids or contain smaller, less dense inner bodies; in addition, the outer membranes of these and other particles may be indistinct or apparently absent. These effects have been shown to result from the level at which these relatively large viral particles have been cut, since serial sections almost invariably reveal both membrane and nucleoid when the plane of section passes through or near the center of the particle (106). At the final stage of development, the virus becomes somewhat larger (approximately 350 \times 200 m μ) and consists of an inner body of variable shape and density enclosed by a double limiting membrane. The structure of the completed virus has been subjected to chemical analysis—using preparations of purified viral particles—which has shown that the inner body, or nucleoid, probably contains both protein and deoxyribonucleic acid (127). The nucleoid has also been found to have

inner and outer zones of markedly different electron density, thus suggesting a definite underlying structural differentiation (49).

In cells infected with vaccinia, fowl pox, and ectromelia viruses, the nuclei generally appear normal (58, 105). Although the mitochondria often are increased in number and may show various stages of degenerative change, they do not seem to participate in viral development (44, 105).

In molluscum contagiosum and Shope fibroma, the stages of viral development and the size and structure of the viral particles are identical to those already described for vaccinia and fowl pox (17, 44), but in these diseases it is obvious that the virus is in some way related to a proliferative or neoplastic type of cellular response. Examination of tissue from cases of molluscum contagiosum has revealed that the strata of epithelial cells resemble those of normal skin. In the basal layer frequent mitoses occur, but cells containing definite viral particles are not seen. Indeed, it is stated that cells with evident viral particles are never found in mitosis (44), an observation in accord with the finding that the division of hepatic parenchymal cells is inhibited in mice following intravenous infection with ectromelia virus (121); that cells infected with herpes simplex virus promptly cease their mitotic activity (153, 154); and that the epidermal cells with morphologic stigmata of infection, in viral papillomas of human skin, lose their capacity for mitotic proliferation (26). In the spinous layer, however, many cells show developmental forms of virus in the cytoplasm, chiefly the single-membrane type with eccentric nucleoid which apparently evolves in the manner previously described. In addition, nuclear changes not yet discovered in vaccinia, fowl pox, ectromelia, or Shope fibroma may be observed (44). These changes consist of dense, irregular deposits of granular or reticular material, not unlike those seen in the nuclei of cells infected with Asian strains of influenza virus (119); their significance is currently unknown. Lastly, in cells of the granular and keratinous layers, large aggregates of mature viral particles occur, and it is these cells which contain the well-known Henderson-Patterson molluscum inclusion bodies. The mature particles of molluscum contagiosum virus may show a small, very dense granule, approximately 15 $m\mu$ in diameter and surrounded by a pale halo (8, 9, 44); a similar structure has been illustrated in the virus of Shope fibroma (44).

The examination of tissue from rabbits infected with Shope fibroma virus (17) has disclosed cellular changes and developmental forms of virus already discussed. In addition, it should be noted that typical cytoplasmic aggregates of granular "viroplasm," associated with partially formed membranes or completed single-membrane particles which show a nucleoid, have been observed in cultures of rabbit fibroblasts infected with this agent (13). The relation of developing virus to the various cellular components of the neoplasm that it induces remains, however, to be determined.

Mention should also be made of the connection between myxoma and Shope fibroma viruses. Myxoma undoubtedly may be classified with the pox viruses because of its size and morphologic appearance, even though, like the

fibroma virus, it differs from vaccinia and fowl pox virus in its sensitivity to ethyl ether (52). Moreover, an antigenic relation between fibroma and myxoma virus has been demonstrated by studies in which inoculation of rabbits with the former agent was found to produce effective protection against the latter (53).

Certain unusual subcutaneous tumors occurring spontaneously in rhesus monkeys show histologic features—notably acidophilic, cytoplasmic inclusion bodies—which suggest a viral origin (14). A virus that is believed to be a member of the so-called pox group is reported to have been isolated from these tumors (37).

Myxoviruses.—One of the most interesting morphologic features of the myxoviruses, as observed among the members that have been reasonably well studied with the electron microscope thus far, is that they can be structurally identified only at the surface of infected cells (69, 111, 119). In infections caused by representatives of the influenza group—which have received the most attention—the viral particles can generally be classified as spherical and rod-shaped, or filamentous, forms (111). The spherical forms appear to differentiate at or near the cellular surface. As they move out through the cell wall each particle acquires an internal body (20 to 22 μ in diameter), a sharply defined membrane (40 to 45 μ in diameter), and a diffuse outer coat, or layer, which brings the average over-all dimension to approximately 70 μ (111). The rod-like, or filamentous, forms seem to develop by extrusion, and often become so long as to become readily visible in the dark-ground microscope (72) or in the light microscope after staining by various procedures (86). On cross-section the filaments show essentially the same diameter as the spheres, but they are devoid of the internal structure which is so characteristic of the spherical forms. These important structural features of the spheres and filaments, as observed in sections of infected cells (111), have been confirmed by other studies in which purified preparations of viral particles were sedimented by centrifugation, and sections of the pellets thus obtained were examined electron microscopically (21). The filamentous forms, which tend to predominate in newly-isolated strains of influenza virus (38), have been found on direct chemical analysis to contain less nucleic acid (RNA) than the spherical particles (34) and to be rapidly digested by trypsin (165) without showing evidence of the ribonuclease-resistant material which regularly can be demonstrated in the spheres (166). It has also been noted that sonic disruption of filaments is followed by a rise in hemagglutinin titer, but no increase of infectivity (42); nor is infectivity significantly altered by selective osmotic dissolution of the filamentous forms (35, 36). These findings, in conjunction with the observation that the infectivity of influenza virus is directly related to its content of nucleic acid (1, 2), lead to the conclusion that the filamentous form is either largely or completely non-infective. Further support for this hypothesis is supplied by studies of the biological and physical properties of filamentous virus, which indicate that the infectious property of a filament, when it exists, is probably confined to a small

locus where there is an enrichment of viral-type nucleic acid (3, 4). It seems clear that such loci, situated at the free ends of filaments, may occasionally give rise to typical spherical infectious units by a process similar to "budding" (4) and, in fact, there is some evidence gained through electron microscopy that this does occur (119). But it is equally certain that the characteristic spheres do not arise primarily by segmentation of the filamentous form (111, 119).

Influenza virus has been found to contain lipid and polysaccharide constituents, as well as protein and about 0.8 per cent RNA (56). Treatment with ether causes disintegration of the virus into two major particulate components (73). One component has a particle size of about 12 $m\mu$ (74), is composed of ribonucleoprotein, and constitutes the so-called soluble ("s" or "g") antigen; the other is a mucoprotein, larger but less well-defined in size, which represents the hemagglutinin (55). Extensive studies with another of the myxoviruses (fowl plague) have yielded similar results and have permitted a tentative conclusion that the spherical viral particle consists of an outer zone composed of several mucoprotein hemagglutinin units bound together by lipid material, and an inner area containing about six units of s-antigen (138). Electron micrographs of centrally-sectioned particles, with adequate resolution and contrast, actually have revealed that the inner body of influenza virus appears to consist of 4 to 8 dense granules, 10 to 15 $m\mu$ in diameter, in agreement with the postulated structure (119).

Although cells infected with influenza or fowl plague virus fail to show any recognizable viral particles in the depths of the cytoplasm or in the nucleus (69, 111), the presence of specific viral antigens may be detected soon after infection by immuno-fluorescent staining procedures (168). When fluorescein-tagged antibody specific for s-antigen is used, the nuclei are the first structures to exhibit fluorescence (87). As the infection proceeds, fluorescence increases in the nucleus and begins to appear in the cytoplasm. Finally, the entire cell may become brightly fluorescent (32, 138). It may be inferred that the nucleoprotein components of the virus are synthesized in the nucleus; moreover, participation of the nucleus in the mechanism of viral propagation may possibly be reflected in a structural alteration which has been observed most frequently in endodermal cells of chicken embryo chorioallantoic membrane infected with newly-isolated strains of Asian influenza virus, but which also has been seen occasionally in cells infected by well-established egg strains, viz., PR8 and Lee (119). This structural abnormality consists in the appearance of dense, irregular aggregates of reticular material often accompanied by decrease in the electron density of other parts of the nucleus, so that "clear" areas develop. However, it should be emphasized that viral multiplication may occur in cells that look entirely normal or healthy (119). The nucleoprotein components of the virus arising in the nucleus presumably are transferred to the cell surface, where the outer units (lipid and mucoprotein) are being produced. Hypothetically, the major portion, or even all, of the peripheral cytoplasm of an infected cell could be so altered as to become

engaged in the manufacture of viral hemagglutinin and lipid while still retaining its structural integrity. The formation of a spherical viral particle would then involve the enclosure of migrating viral nucleoprotein by structurally normal (but chemically modified) cytoplasm as the particle emerged from the cell. Also, hypothetically, a defect or irregularity in the mechanism could result in continued formation of the external components of the virus without the regulating effect imposed by incorporation of nucleoprotein, thus leading to the extrusion of filamentous elements with the antigenic properties and specificity of viral hemagglutinin, as well as the osmotic characteristics of the cytoplasm, but lacking or deficient in nucleic acid. In this connection it may be pointed out that in the hemadsorption phenomenon (167) with influenza virus, erythrocytes may be bound to the surface of infected cells either by "bridging" with viral particles (71, 119) or by direct adhesion (71), both types of binding being prevented by specific antibody. This phenomenon is consistent with the conclusion that immunologically specific changes occur at the surface of the host cell following infection by influenza virus.

Little additional information is yet available concerning alterations in cellular structure induced by myxoviruses. Studies of ferret respiratory cells infected with influenza virus disclosed cytopathic changes which could not be related directly to viral development (70). Nor could any specific morphologic connection be established between the multiplication of Newcastle disease virus and cytologic changes occurring in Ehrlich ascites tumor cells infected with this agent (5).

Psittacosis-lymphogranuloma venereum group.—In cells of chicken embryo chorioallantoic membrane infected with meningoepneumonitis virus, the nuclei generally appear normal, although they may be displaced or compressed by accumulations of developing viral particles, which characteristically appear in large aggregates (59). Such aggregates are often contained within cytoplasmic vacuoles, some of which may show evidence of a peripheral membrane (159). Other cells exhibit more widely dispersed particles with random distribution. A similar picture has been observed in chicken embryonic tissue infected with a bovine strain of virus belonging to this group (159), as well as conjunctival tissue obtained from patients suffering from trachoma (99, 100, 101). The viral bodies occurring in individual cells are round or oval and have usually been found to vary considerably in size, ranging from 200 to 500 μ , presumably because of differences in the stage of development. There also are obvious variations in the internal structure of such bodies, the significance of which remains conjectural. In general, the smaller bodies, or particles, appear relatively dense throughout, or consist of a dense central body and a lighter peripheral zone, with the suggestion of a limiting membrane. The larger ones frequently are finely granular, show a definite limiting membrane, and may contain a "nucleoid," thus resembling the pox viruses, as noted below. The collections of particles, especially those occurring as "colonies" within cytoplasmic vacuoles, often appear to be

embedded in an amorphous ground substance, or matrix, whose nature has not been determined. Observations of viral development in the endodermal chorioallantoic cells of chicken embryos infected with meningoencephalomyelitis virus and treated with penicillin, have revealed that the antibiotic interferes with both matrix formation and the segmentation of particles. As a result, aberrant forms develop and these undergo gradual disintegration (160).

Infected cells generally show variable degenerative changes in mitochondria and disorganization of elements of the endoplasmic reticulum; in addition, large deposits of a dense, amorphous, osmiophilic material—presumably some sort of lipid—are frequently encountered. However, these structural abnormalities are not in any way specifically related to infection by this group of viruses, since they are often seen in cells infected by other viral agents, as well as in normal cells that have reached the end of their life span and are undergoing spontaneous degeneration (113).

Enteroviruses.—Relatively little is known about the morphologic changes, as revealed by the electron microscope, in cells that have been infected by members of this large and important group of viruses. Early studies of monkey kidney cell cultures inoculated with type 1 poliovirus disclosed collections of osmiophilic particles, whose size was compatible with that of the virus, occurring in nuclei most commonly in areas near the nucleolus. It was also noted that infected cells exhibited shrunken, lobulated nuclei, margination of nuclear chromatin, abnormal banding of mitochondria, osmiophilic (lipid) droplets in the cytoplasm, and cytoplasmic areas in which the normally granular structure appeared finely filamentous (135). None of these changes, however, could be definitively associated either with the virus itself or its effects on the cell, and indeed all of them have been observed in degenerating cultures of normal cells. Subsequent attempts to delineate more clearly the relationship between cellular changes and the stage of viral infection by following the sequence of events during a one-step growth cycle of type 1 poliovirus in suspended cultures of monkey kidney cells, unfortunately did not succeed either in demonstrating the virus within cells or in disclosing cytopathic alterations which could be specifically ascribed to its action (77). More recently, however, type 1 poliovirus has been clearly visualized in human amnion cells, strain FL, at a late stage of infection (157). Such cells were found to show non-specific cytopathic changes consisting primarily of cytoplasmic breakdown and nuclear lobulation. The virus was located exclusively in the cytoplasm, where it occurred as large crystalline aggregates of spherical particles. Individual particles measured $24\text{ m}\mu$ in diameter with an inner dense zone of $16\text{ m}\mu$ diameter and a less dense central core $4\text{ m}\mu$ in diameter. Allowing for shrinkage in preparation of the specimen, the particle size closely approximates $27\text{ m}\mu$. Almost exactly similar observations have been recorded for type B-5 Coxsackie virus. In HeLa cells examined four to five days after inoculation with this virus, viral crystals composed of spherical particles with $25\text{ m}\mu$ center-to-center spacing have been encountered (118), the actual diameter of the particles corresponding to $28\text{ m}\mu$ as calculated from

pseudo-replicas of purified preparations (31, 94). Certain morphologic changes—shrinkage of the cell, pyknosis of the nucleus, condensation and vacuolization of the cytoplasm, and disruption of mitochondria—were frequently found to be associated with intracellular virus, but they could not be considered pathognomonic, since similar alterations have been encountered in uninfected cultures of HeLa cells (113). Crystals of Coxsackie B-5 virus, resembling those seen in HeLa cells, have also been reported in infected cultures of the FL strain of human amnion cells (54). In this system also, the cytopathic effects of viral infection, as evidenced by nuclear deformation, vesiculation of the endoplasmic reticulum, and swelling and disorganization of mitochondria, failed to include any abnormalities of cellular fine structure which could be called typical or specific. There is virtually no information available to date on changes that may occur in the intact tissues of man or animals infected with enteroviruses. Examination of pancreatic tissue removed from adult mice after inoculation with a group B strain of Coxsackie virus did not disclose any characteristic anomalies (130).

Measles virus.—HeLa cells infected with measles virus in tissue culture have been discovered to contain unusual nuclear inclusions characterized by ordered arrays of filamentous structures (78). The individual filaments in these crystal-like bodies have a length of about 0.2 to $1.0\ \mu$ and a width of approximately $20\ m\mu$. It is not probable that they represent mature measles virus, but their constant association with measles infection seems to relate them to the infectious process.

VIRUSES DEVELOPING PRIMARILY IN THE NUCLEUS

Adenoviruses.—Early studies of this group of viruses indicated that they develop primarily in the nuclei of infected cells, where they may occur in ordered or crystalline arrays (64, 82). Subsequently, it has been ascertained that the viral particles appear to differentiate from aggregates of dense granular or reticular material within nuclei, and that among the 18 human and 5 simian serotypes which have been identified thus far (133), strains of types 3, 4, 7, and 8 commonly produce numerous crystals, whereas this phenomenon is less commonly exhibited by types 1, 2, 5, and 6 (114, 119). The viral crystals may attain considerable size and thus become visible in the light microscope. Their structure has been examined by the model reconstruction technique, using serial sections cut at different angles as the reference data, and the lattice appears to be of the cubic body-centered type (88). Individual viral particles are approximately $70\ m\mu$ in diameter, and in cells fixed by osmium tetroxide they occur predominantly in two forms: one is dense with little visible internal structure, and the other, which constitutes the majority of the particles, is less dense and contains an internal body approximately $25\ m\mu$ in diameter (109). Fixation with formalin alters the appearance of the virus so that the internal body approaches a diameter of $60\ m\mu$ and the particle exhibits a sharply defined peripheral membrane (119). A somewhat similar picture is seen when the virus is fixed with osmium and then stained

with uranyl acetate (66). Although the particles seen in sections of osmium- or formalin-fixed tissue are usually round or ovoid, they have been found to be polyhedral when viewed in suspension after fixation by osmium (163), or osmium fixation followed by treatment with 1.0 per cent phosphotungstic acid (164). Moreover, when particles are examined at high resolution, using a negative staining method with phosphotungstic acid (33), it has been determined that they are probably icosahedral in shape and have a shell composed of 252 subunits, each of which presumably is a protein molecule (68). In all likelihood, the nucleic acid moiety of the virus is DNA, as suggested by cytochemical studies in which Feulgen-positive structures have been observed to develop in the nuclei of infected cells (28, 65, 85), and shown more definitely by the repeated observation, using alternating thick and thin sections of identical cells, that the same viral crystals seen electron microscopically are also strongly Feulgen-positive when examined cytochemically (27). Association of DNA with the synthesis and probably with the structure of adenovirus is supported, in addition, by the results of studies which have shown that the biochemical alterations found in cells infected with adenovirus are intimately related to the infectious process (61), and that type-specific viral antigen occurs in the characteristic intranuclear structures composed of viral particles (29).

Cells infected with certain strains of type 5 adenovirus, when viewed in the light microscope, have been found to contain numerous large oblong or bar-shaped crystals (85). In the electron microscope these crystalline deposits have been shown to develop only in the nucleus and to occur exclusively in cells that actually are infected, as judged by the concurrent presence of viral particles (112). The crystals are composed of protein and are devoid of nucleic acid; many appear hexagonal in cross-section and some are extremely large, approaching $30\ \mu$ in length. At high resolution the fine structure of this crystalline protein has been visualized and found to show an unusually wide molecular spacing of 400 Å (112). Cytochemical and immunofluorescent staining procedures have demonstrated that it has basic properties (112), differs from normal nucleohistone (112), and does not contain type-specific viral antigen (29). The significance of this unusual protein currently remains obscure.

The structural changes seen in cells infected by adenoviruses (83, 109) consist initially of aggregates of dense reticulum at multiple foci in the nucleus. Elsewhere in the nucleus the rather coarsely granular reticulum seen normally may be altered to a greater or lesser extent so as to become more finely granular and "clearer" in appearance. The nucleoli rarely are altered and show no consistent orientation with respect to the developing virus. In the light microscope the aggregates of reticulum are at first acidophilic and Feulgen-negative, whereas at a later stage, when clusters or crystals of virus are developing in association with them, they become basophilic and Feulgen-positive (28). This spatial relationship, together with the observation that the reticulum diminishes as the virus increases, suggests that the reticular

material contributes somehow to propagation of the virus. As viral multiplication proceeds, the nuclei may exhibit extraordinary numbers of particles, whether or not in crystalline array, and their release seems to depend on rupture of the nuclear membrane. When nuclear rupture has occurred, large numbers of particles may be found in the cytoplasm—as well as crystals of non-viral protein in the case of type 5 adenovirus—and this probably accounts for the observation in experiments using the fluorescent antibody technique that many cells with specific nuclear fluorescence also show much weaker but definite cytoplasmic fluorescence (126). As the final stage in the cycle of infection with this group of viruses, it seems probable that virus escapes from both nucleus and cytoplasm as the result of a degenerative process which leads to cellular disintegration (109, 119).

The changes in nuclear reticulum already referred to may be considered to be typical of, but not necessarily specific for, infection by adenoviruses. In addition, it should be mentioned that some types of adenovirus, particularly type 3, as studied thus far, excite the development of peculiar laminated membranal structures within nuclei (109). This sort of structure has never been encountered in normal cells and has not yet been observed in cells infected by other viruses. For the time being it may be considered as some sort of specific, although unexplained, effect. Reduplication of nuclear membranes has also been noted (63), as well as has the occurrence of parallel membranes within nuclei (109), but these phenomena may represent a basic and important type of response to infection by a variety of viral agents. So far as cytoplasmic changes are concerned, it should be emphasized that adenoviral infection causes only those non-specific effects which have previously been described in cells undergoing spontaneous degeneration.

Herpes simplex virus.—The earliest effects of cellular infection with herpes simplex virus, as noted by both light (12) and electron (104, 120, 129, 152) microscopic studies, consist of nuclear changes in which the nucleoli undergo fragmentation or transposition, accompanied by an accumulation of reticular material on the inner surface of the nuclear membrane. This material, which has been referred to as "marginated chromatin," appears to consist of both nuclear and nucleolar elements; indeed, structures considered to be nucleoli have been found flattened against the nuclear membrane in many instances (104). At this stage also the nuclei often appear somewhat swollen and irregular in shape, and the remainder of the nuclear matrix is more finely granular and lighter, i.e., less electron-diffractive, than usual. These early and typical changes, although indicative of infection, may not be accompanied by the presence of recognizable viral particles, possibly because such particles, even though they may already exist at several small loci in the nucleus, are not included in the plane of section. As viral propagation proceeds, numerous so-called "single membrane" forms appear, chiefly in association with the peripheral reticular deposits already mentioned, but also dispersed at random in many instances. These particulate forms consist of a central body approximately 40 μ in diameter, surrounded by a sharply

defined membrane about 90 $\mu\mu$ in diameter. With certain strains of virus that have been found to induce the formation of crystals of single-membrane particles (115), the crystals consistently have been seen apposed to aggregates of relatively uniform granules $\sim 20 \mu\mu$ in diameter, which can easily be distinguished from other components of the marginated reticulum. Moreover these crystals may extend into the granular aggregates in such a way as to suggest that the former are replacing the latter at template sites. The single-membrane particles while still within the nucleus acquire a second membrane and become approximately 130 $\mu\mu$ in diameter; at the same time the density of the central body becomes greatly increased. The construction of this second membrane is apparently the result of a remarkable process that also seems to be part of a mechanism for releasing the virus from the nucleus without loss of nuclear integrity. This process involves reduplication of the nuclear membrane itself, together with formation of scattered membranal structures inside the nucleus. These latter structures presumably arise at sites removed from the primary template areas, since double-membrane particles have not been encountered within either the granular aggregates or the crystals of single-membrane forms where the internal body and initial membrane are believed to differentiate. In a system of this kind it might be expected that the outer membrane, as it develops, would occasionally enclose more than one single-membrane particle and, indeed, "twin" particles consisting of two single-membrane forms surrounded by an outer membrane have been frequently observed in cells infected with both herpes simplex (119, 120) and herpes B (129) viruses, as well as varicella virus (162). Reduplication of the nuclear membrane seems to occur in such a way that completed (double-membrane) particles escape from the nucleus by a mechanism in which new membranes are laid down successively behind the virus as it passes into the cytoplasm. Evidence for this ingenuity on the part of the cell is provided by the frequent discovery of viral particles that are situated between the multiple nuclear membranes (120). In the cytoplasm, the virus is usually seen to be located in walled vacuoles, whose limiting membranes are probably derived from the nucleus, although participation of the endoplasmic reticulum in their formation cannot be entirely excluded. Rupture of these vacuoles at the cell surface can result in extrusion of virus without disruption of the cell (120). The cytoplasm of infected cells has also been found to contain numerous lamellae, many of which exhibit pore-like structures, but except for these and the walled vacuoles containing virus, the changes are not in any way specific or distinctive.

It is of interest that cells infected with herpes simplex virus can be construed on morphologic grounds alone to possess the capacity to elaborate and excrete newly-formed virus without the necessity of either nuclear or cytoplasmic breakdown as a release mechanism, and this actually has been found to be the case in correlated studies of viral multiplication and its effects as observed in the light microscope (131, 139, 153).

Varicella virus.—Cells of tissue obtained from fresh vesicles in cases of

chickenpox, as well as human embryonal cells (predominantly fibroblasts) in tissue cultures infected with varicella virus, have been found to show structural changes and developing forms of virus (162) that closely resemble those seen with herpes simplex (104) and herpes B (129) virus. In early stages of infection the nuclei often display marginated chromatin, scattered collections of rather dense granular material, and areas of decreased electron density. At this time many cells contain no evident viral particles. Later, however, both single and double membrane forms are observed in the nucleus, and double membrane particles can be found between nuclear membranes, suggesting the release mechanism postulated for herpes simplex virus (120). In the cytoplasm large numbers of complete (double membrane) particles are seen in vacuoles which have a well-defined limiting membrane. The particles have an inner body (30 to 40 μ in diameter), an inner membrane (70 to 110 μ in diameter), and an outer membrane (100 to 160 μ in diameter), thus being similar in size as well as internal structure to herpes simplex virus (104). Extracellular particles may be somewhat larger, approaching 200 μ in diameter.

Salivary gland virus.—The alterations observed in cells infected by human and murine strains of salivary gland virus (89, 90) resemble, in certain respects, those induced by herpes simplex virus (104, 120), and in others, those associated with a virus-like agent that has been observed in the Lucké renal adenocarcinoma of frogs (51). Nuclear changes may include margination of reticular substance, although the nucleoli usually remain intact. The principal nuclear abnormality, however, is the appearance of a large central deposit of dense, strand-like, coarsely granular material, which enmeshes large numbers of viral particles, some of which consist of a central body and single limiting membrane, whereas others exhibit a dense central body and double membrane. The larger, double-membrane particles (referred to as target-like forms) have been found to range from 120 to 180 μ in diameter. Reduplication of nuclear membranes and intranuclear membranal structures have not been described, although viral particles have been observed between nuclear membranes, as has been noted with herpes simplex virus (120). In the cytoplasm large vacuoles with a well-defined limiting membrane have been seen repeatedly; these vacuoles often enclose large numbers of target-like viral particles. The cytoplasm also may exhibit numerous large, dense spherical bodies whose significance is uncertain. Some of these contain viral particles. It seems probable that future work will disclose an even closer resemblance between salivary gland virus and herpes simplex virus—so far as their developmental cycle and effects on cellular fine structure are concerned—than has been discovered to date.

BACTERIAL VIRUSES

Investigation of the changes in fine structure that may occur in bacterial cells as the result of viral (bacteriophage) infection has been hampered by technical factors, by the organizational characteristics of bacterial proto-

plasm, and by differences in the nature of the infecting agents, e.g., vegetative as compared with temperate phages. In early electron microscopic studies of *E. coli* infected with T-even phage, it was evident that cell preservation by the methods of fixation then employed was not optimal, although sufficient information was obtained to justify the conclusion that filamentous structures, which were typically found in "nuclear sites" or "nucleoids" of these bacteria, resembled morphologically the nucleoprotein elements seen in sections of free phage particles (91). Improvements in technique have since made it possible to study the internal features of normal and infected bacteria whose architecture has been much less affected by the procedures of fixation, embedding, and sectioning (136). Broadly speaking, the bacterial cytoplasm has been shown to consist mainly of close-packed granules from 10 to 15 μ in diameter, which correspond to the component that contains most of the RNA (137) and probably are equivalent to the "Palade granules" of vertebrate cells (125). The DNA-containing nucleoplasm, on the other hand, has a finely filamentous appearance (136). No differentiating membrane between "nucleus" and "cytoplasm" has been described, and thus there is presumably a close association between DNA and the cytoplasm. Whether and to what extent DNA may exist in the cytoplasm is currently uncertain. In any case, it would seem that the relationship between DNA, cytoplasmic components, and the cytoplasmic membrane has a simpler organization in bacteria than in higher cells (57).

Electron microscopic examination of bacteria following infection by vegetative phage has shown that first the bacterial nucleoids migrate slowly toward the cell wall and there form marginal vacuoles. From these vacuoles a phage-DNA pool composed of extremely fine fibrils at the very limit of resolution and contrast of the electron microscope (30 to 50 Å) grows out and moves toward the center of the cell. Soon thereafter dark bodies with the polyhedral shape of phage heads are seen in the sections, and these continue to increase in number until 30 or more may be recognized in a single section (80). No such bodies appear in cells exposed to chloramphenicol at the time of infection as long as the drug is present; but they begin to be formed soon after removal of the drug. This finding indicates that protein synthesis is necessary for condensation of the phage DNA into the first form of immature phage. Unfortunately, several limitations in technique have restricted the accumulation of additional morphologic data. Since the thickness of sections (about 50 μ) is more than half the diameter of phage heads, the phage membrane becomes visible only occasionally when it lies exactly perpendicular to the plane of section. Also, formation of the tail has rarely been observed, because known methods usually do not give enough contrast to the tail to make it visible in micrographs (80); however, tails have been visualized in some preparations (98). It is of great interest that although infection by vegetative phage may quantitatively alter the fine structure of bacteria, chiefly with respect to increase in the amount of fibrillar material associated with DNA, the actual components of both cytoplasm and nucleoplasm, as seen in normal cells, show no specific changes. Nevertheless, since it has been

shown that phage infection may induce the formation of polyamines, e.g., spermidine and putrescine, which are simpler than histone or protamine, but capable of reacting with free acidic groups of DNA (6), it may be that the resulting chromatins eventually can be distinguished by appropriate methods of fixation and staining, thus providing a means for detecting DNA-containing plasms of different chemical composition (79).

Nothing is known at present about the effects of temperate phages on the morphologic structure of bacteria harboring or infected with these agents.

INSECT VIRUSES

The larvae of many insects are subject to viral infections, which generally have been classified into two major types: polyhedral diseases, and capsule (granulosis) diseases (16). The polyhedral diseases, or polyhedroses, derive their name from the observation that large polyhedral bodies are formed in certain tissues of the host, where some characteristically occur within the nuclei of infected cells, whereas others are seen only in the cytoplasm. The capsule diseases, or granuloses, are so called because the viral particles, which develop only in the cytoplasm, appear to be enclosed in a capsule or granule. No polyhedral bodies are associated with the granuloses. A third group of insect virus diseases has also been described in which the infecting agents more closely resemble those found in virus diseases of animals and higher plants (149). The viral particles are not occluded by crystalline protein, as in polyhedra, nor enclosed in capsular membranes, as in granulosis, but rather are found free in the cytoplasm, where they may form large crystalline aggregates (150, 170) not unlike those observed with adenoviruses (109).

The examination of cells soon after infection with nuclear polyhedral viruses has revealed swelling of the nuclei and the appearance of irregular, dense accumulations of a reticular substance, or chromatin, in the nucleoplasm (22, 39, 75, 146, 161, 171). Rod-shaped viral particles emerge in the vicinity of, and may actually derive from, this reticular substance (22, 39, 75, 144, 145, 146, 161); they vary in size, ranging from 20 to 50 μ in width and 200 to 400 μ in length (24). Although there is some difference of opinion, it seems that these rods are next enveloped by membranal structures that enclose from one to eight particles in a single membrane. Lastly, the majority of the membrane-enclosed particles are occluded by a crystalline protein to form the polyhedra. Study of the polyhedra in thin sections has shown that the protein molecules are 65 to 71 Å in diameter and approximately 180 Å long (39, 107). The lattice of the crystal is not disturbed by the membrane-enclosed viral particles, which, in serial sections of *Porthetria dispar* virus, were found to average 20 μ in diameter and 280 μ in length (108). Polyhedra arising in the cytoplasm have been insufficiently studied but appear to develop by a process somewhat similar to that just described (149, 172). It has been claimed that viruses of the nuclear polyhedroses contain only deoxyribonucleic acid, whereas those of the cytoplasmic polyhedroses contain only ribonucleic acid (149).

In cells infected with granulosis viruses the cytoplasm shows round or

oval structures about $150 \times 500 \text{ m}\mu$ in size (23). These consist of the viral particles embedded in a protein matrix. The development of the virus appears to resemble that of polyhedral types in that rod-like particles seem to arise from areas of altered cytoplasmic reticulum (149), to be enclosed by membranes, and finally to be surrounded by protein (23). However, unlike the polyhedra which show many bundles of two or more rods, the granules contain—except for an occasional bundle of two—only a single rod. The nuclei of the infected cells usually appear normal. It is interesting that although polyhedral and granulosis viruses compete for cells so that prior infection with one tends to exclude the other, double infections have been induced by properly adjusting the time and size of inocula (24). Doubly-infected cells show typical polyhedra in the nucleus and granulosis forms in the cytoplasm.

The prototype of insect viruses which are not associated with any kind of capsule or occluding protein, is the crystallizable, iridescent virus found in larvae of *Tipula paludosa* (147, 148). This virus multiplies in the cytoplasm of cells whose nuclei are not morphologically altered (148). Participation of the nuclei in the mechanism of viral reproduction, however, has not been excluded. The viral particles may form large intracellular crystals and grow to such enormous numbers in the late stage of infection that they constitute one-fourth of the larva's dry body weight. The virus can easily be extracted from diseased larvae and purified by differential centrifugation. The purified particles form iridescent crystals on centrifugation, and spontaneously when allowed to stand at low temperature. Individual particles have a diameter of approximately $130 \text{ m}\mu$ and exhibit an hexagonal contour in thin sections. They contain deoxyribonucleic acid which comprises about 15 per cent of the viral mass (149).

PLANT VIRUSES

The remarkable recent advances in knowledge of the morphologic, biologic, and chemical characteristics of plant viruses unfortunately have not been paralleled by information concerning their mode of development in tissues and their effects upon cellular structure.

In cells infected with tobacco mosaic virus, rod-shaped particles presumed to be the virus have been visualized only in the cytoplasm, where they appear to be associated with the endoplasmic reticulum (93). It is now believed that chloroplasts are not directly associated with reproduction of the virus, and that the disintegration or disorganization they may show in infected cells is a secondary event which is unrelated to viral propagation (93). Similarly, in plants infected with barley stripe mosaic virus, structures resembling viral particles have been encountered only in the cytoplasm of cells whose nuclei and chloroplasts were not significantly altered (141). A method has been described for improving the electron density of tobacco mosaic virus in thin sections by staining with phosphotungstic acid (140).

VIRUSES ASSOCIATED WITH TUMORS

The structural alteration of cells in tumors that are or may be associated with viral agents is a subject which largely lies outside the bounds of this

limited review. Moreover, at this stage in our knowledge concerning the etiologic role of viruses in neoplasms, it would be impossible to give a single example in which direct and specific evidence has been obtained of the mechanism by which viral infection induces the formation of either benign or malignant tumors. Until such evidence is forthcoming, any attempt to construe morphologic features of tumor cells in terms of their actual relation to viruses would represent merely an exercise of the imagination, hopefully tempered by logical interpretation of the existing data.

It may be pointed out, however, that several different morphologic varieties of subcellular particles have now been consistently identified in association with a number of malignant neoplasms which occur both in lower animals (including cold-blooded species) and in birds (19). Although similar findings have not yet been recorded in human cancer, certain benign tumors of man seem clearly to result from viral infection, as already noted in the case of molluscum contagiosum (see pox viruses) and less definitely shown in laryngeal papilloma, common warts, and venereal warts (19).

So far as a relation between viral infection and tumor induction is concerned, it has been known for many years that Shope papilloma induced in domestic rabbits may fail to contain virus, as demonstrated by conventional methods (142, 143), even though the animals develop virus-neutralizing and complement-fixing antibodies in their sera (81). The observation is consistent with the finding that this and other tumors may vary widely in their content of virus (15). Recent electron microscopic studies have disclosed the papilloma agent within cells of the tumor, where characteristic particles (25 to 35 $m\mu$ in diameter) are seen first in nuclei, in which they may differentiate from altered nucleoli (103, 156). Investigation of different areas of the neoplasm has suggested that the age of cells, determined by their position in the Malpighian layer and by the extent of keratinization, can be correlated with viral development, and that the virus possibly acts either indirectly to cause hyperplasia in cells not themselves infected, or directly to stimulate cell proliferation at the germinal layer before viral multiplication becomes evident by electron microscopy (156). Other evidence to support these findings is provided by studies in which viral antigen, as revealed by fluorescent antibody, has been found principally or exclusively in the keratohyaline or keratinized layers and could not be detected in the deeper proliferating epithelial cells (122). In addition, the results of experiments in which either the keratinized or the proliferating layer of papillomas was selectively destroyed by microcautery have indicated that the infective virus is situated mainly, perhaps entirely, in the keratohyaline and keratinized layers (123).

In Rous sarcoma the viral agent has been readily demonstrated in thin sections of tumor tissue from both young chickens and chicken embryos (18). Mature particles are about 75 $m\mu$ in diameter and have a definite structure consisting of an outer membrane and a dense inner body, or nucleoid, about 35 $m\mu$ in diameter. Examination of the particles after treatment with ribonuclease indicates that the nucleoids may contain ribonucleic acid (50). Development of the virus has been observed solely in the cytoplasm, where

it seems to differentiate from dense, irregular, granular masses, which possibly are derived from the ribonucleoprotein granules (125) of the endoplasmic reticulum (18). The viral particles are frequently seen on the surface of cells, as well as in large cytoplasmic vacuoles which have a well-defined membrane; it seems likely that virus is released from these vacuoles by rupture at the cell surface in a manner analogous to that postulated for herpes simplex virus (120). It is important to note that the ultrastructure of the tumor cells is somewhat different from that of normal cells, but that the nuclear and cytoplasmic changes are similar to those seen in many other types of cancer, and therefore must be considered non-specific (19). Electron microscopic examination of Rous sarcoma tissue, in combination with infectivity studies, has shown no evident connection between the number of demonstrable viral particles and the rapidity of tumor growth, but a reasonably good correlation between particle number and infectivity titer is seen (18). The definite lack of relationship between the rate or extent of tumor production and the presence of visible viral particles may serve only to emphasize one of the most difficult problems in electron microscopy, the so-called "sampling error" which was mentioned earlier, and may actually be the result solely of quantitative variations in the amount of virus (15); but information obtained by different methods suggests other interesting alternatives. For example, it has been shown in cultures of chicken embryonic tissue infected with the Rous virus that the monocytes or macrophages which apparently arise from fibroblasts by transformation no longer are capable of producing virus, while the typical giant cells release large amounts of virus although they are no longer capable of dividing (62). These results have raised the question whether the cellular transformation may be mediated by latent viral infection or by the action of substances, possibly mucopolysaccharides, that are liberated in the medium of infected cultures (62).

The oncogenic agent now known as SE polyoma virus induces multiple tumors in mice (151) and hamsters (48), causes cytopathic effects in tissue culture (47), agglutinates erythrocytes (46), and elicits specific antibodies (132, 134). Free viral particles observed in metal-shadowed preparations from tissue cultures were found to be 40 to 45 μ in diameter (76). Electron microscopic examination of infected cells in tissue culture has shown large numbers of particles approximately 30 μ in diameter, primarily located in the nuclei (10, 20, 41) where viral antigen first becomes detectable by fluorescent antibody staining (67). The particles appear to develop from intranuclear accumulations of dense chromatin and may form crystalline arrays (20). Crystalline aggregates have also been seen in the cytoplasm, and many particles may be found on or between cell surfaces (10). In addition, somewhat larger forms (40 to 60 μ in diameter) whose relation to the smaller ones is obscure, have been observed in the cytoplasm. Similar particles presumed to be virus have also been demonstrated in cells infected by the Mill Hill polyoma agent (43). A DNase-sensitive, RNase-resistant infectious component has been extracted from the SE polyoma virus, suggesting that its nucleic

acid is DNA (40). Still another "polyoma" agent has been described (92) whose origin differs, however, in that it was recovered from C3H mouse mammary tumor, rather than from leukemic tissue of AK mice, as was the case for both the SE and Mill Hill strains. Like these latter strains it induces multiple tumors, is cytopathogenic in tissue culture, and causes hemagglutination. Preliminary electron microscopic studies have shown large numbers of extracellular particles, about 110 μ in diameter with a dense central nucleoid, in some but not all of the tumors developing in mice after infection by this agent (92).

Studies of the mouse mammary tumor agent, originally shown by Bittner (25) to be transmitted by the milk of high-cancer-strain females, have now shown fairly definitely that the agent may be classified as a virus whose activity is associated with a particle approximately 100 μ in diameter, as well as with a smaller particle 20 to 30 μ in diameter (102, 155). Tissue cultured fragments of the tumor (84), on electron microscopic examination, show many particles about 95 to 100 μ in diameter, with a small dense nucleoid (in a few particles double nucleoids have been observed). The particles are exclusively extracellular and appear to arise at the cell margin, as in the case of influenza virus. Cytopathic effects associated with growth of the tumor tissue *in vitro* have not been observed, despite the fact that viral multiplication appears to be taking place. In regard to this finding, it may be noted that other viruses, e.g., influenza virus, can propagate in cells which morphologically appear healthy (111, 119), and that cells infected with either influenza or Newcastle disease virus can continue to divide while elaborating new virus, instead of undergoing mitotic arrest (169).

Further consideration of the relation between tumors and viral agents cannot be undertaken in this discussion and the reader is referred to other sources (7) for additional information.

COMMENT

The pathologist has long recognized that viral infection is manifested, in a clinical sense, by cellular degeneration, cellular proliferation, or a combination of these processes. More recently it has become widely apparent, in addition, that some infections may long remain latent or occult, and that certain viruses are capable not only of parasitizing cells as a means of sustenance and procreation, but also of transforming or transducing them genetically. When the effects of a virus upon its host are represented chiefly by damage to or death of susceptible cells, the nature and extent of injury to specific elements of tissue is reflected in more or less reproducible patterns of disease, which may be classified according to signs, symptoms, and laboratory findings, and constitute the so-called infectious diseases of viral etiology. In some of these diseases, as indicated in this discussion, it is now possible morphologically to identify the etiologic agents at the sites of infection and to learn something of their mode of development, as well as their relation to cell structure. Obviously, there are wide differences, but also striking similarities, among them.

Some multiply primarily in the nucleus, others in the cytoplasm, and still others are recognizable only at the cell surface. They vary widely in size, structure, and apparent type of development, but many can already be divided into groups on the basis of easily recognizable characteristics; indeed, fine structure undoubtedly will be an important feature in their future classification. Unfortunately, little has been discovered at the morphologic level to indicate that these viruses cause distinctive or specific alterations of normal cellular components which might aid further in elucidating the mechanisms of their reproduction and cytopathogenicity. It is worthy of emphasis that the few possibly specific changes seen to date—reduplication of nuclear membranes (63), laminated intranuclear structures (109), scattered intranuclear membranes (109, 119)—have all been found in the nucleus. None of the cytoplasmic alterations can be distinguished from those seen in spontaneously degenerating cells.

The list of viral agents known to cause either benign or malignant proliferative lesions is growing rapidly. From the morphologic or structural standpoint, it is evident that they too, like the viruses which produce "infectious diseases," can probably be classified into different groups with common features (19). Also, like the "infectious" viruses, some with similar structure have been found to be associated with different disease patterns, i.e., different types of neoplasms (19). The analogy can be carried still further in the observation that abnormal cytologic changes in the cells of malignant tumors known to be induced by certain viruses are non-specific in the sense that exactly similar abnormalities may be seen in the cells of other cancers (18). Lastly, there is ample evidence that certain tumor-inciting viruses, e.g., Shope fibroma and SE polyoma, produce cytopathic effects and mitotic arrest in tissue culture, and stimulate specific antibody responses. The question then arises: in what way do viruses which have the capacity for causing cell degeneration and death manage to excite cellular proliferation and malignant change? One possibility is certainly the excretion, by infected cells, of substances which mediate mitotic activity and cause cellular transformation (62, 103, 117). A mechanism of this type is known to be responsible for crown gall disease of plants, wherein the inciting bacterium *Agrobacterium tumefaciens* elaborates a tumor-inducing principle that converts normal plant cells to tumor cells in short periods of time (30). Another possibility is the transduction of cells by the virus, which may then either persist in latent or masked form (97) or continue to evolve in a cellular milieu that no longer responds by self-injury. It would seem that some type of actively acquired tolerance would be essential for operation of the latter system.

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ENZYME LOCALIZATION IN BACTERIA¹

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Much of the interest in the location of enzymes in bacteria stems from the success of similar studies of the cells of plants and animals, particularly the identification of the mitochondrion as the locus of respiratory enzymes in liver cells. It has frequently been assumed that the location of function in the bacterial cell would be comparable to that of cells of higher organisms even though the bacterium is one-tenth the linear dimensions and is comparable in size with the chondriosomes of larger cells. This hypothesis predicts a smaller version of the same organelles found in larger cells. Mitchell (1) has pointed out clearly that this hypothesis is untenable if the organelle must be reduced in size to the dimensions of individual macromolecules because the macromolecules of bacteria from which their organelles must be constructed are the same size as the macromolecules of larger cells.

An identification of the locus of a substance within the bacterial cell presupposes a knowledge of the topography of the cell. The best method of establishing this topography that is currently available is a combination of phase-contrast microscopy of living cells to avoid the artifacts that result from traditional methods of fixing and staining and of electron microscopy of thin sections for maximum resolution. Electron micrographs of thin sections of bacteria reveal the absence of much of the complex structure found in the cytoplasm of cells of higher organisms. The central nuclear vacuole is surrounded by cytoplasm that is frequently free of structures that could be resolved with the light microscope (2, 3, 4). The bacterial cytoplasm is filled with spherical dense granules about 200 Å in diameter, comparable to the granules attached to the endoplasmic reticulum of higher organisms; however, no reticulum is evident in bacteria (5). The bacterial cytoplasm does occasionally contain larger structures, some of which resemble mitochondria (4, 6, 7, 8); such inclusions are more frequent in cells from older cultures. The obvious dissimilarity between general structure of the bacterial cell and that of larger cells should caution against the uncritical application of the nomenclature of the organelles of larger cells to the structures observed in, or isolated from, bacteria.

DIRECT CYTOCHEMISTRY

Two general procedures may be used to locate enzymes or other substances within a cell: direct cytochemistry or the observation of individual cells, and analytical morphology, the isolation of specific structures from a population of cells. Although direct cytochemistry is potentially a powerful

¹ The survey of the literature pertaining to this review was concluded in January, 1960.

technique for the location of specific substances within a cell, it suffers from a lack of both specificity and sensitivity. The ideal application of direct cytochemistry to the location of enzymes would take advantage of some physical property of the enzyme. Unfortunately, most enzymes lack distinctive properties other than the specific reaction which they catalyze. Even if the enzyme absorbs light of a specific wavelength (e.g., cytochrome) or reacts specifically with a dye, the limit of sensitivity of current optical methods makes it unlikely that the enzyme could be detected at a specific locus in the cell (9). If the enzyme or specifically stained enzyme has a molar extinction coefficient of 10^5 and is located in a structure 0.5μ thick, the concentration required to absorb 10 per cent of the incident light is approximately 0.01 *M*. Thus, to be directly detected, the specific enzyme must reach a concentration in that structure that equals or exceeds the total protein concentration of the cell. Sensitivity could be improved if several molecules of dye could be specifically bound, but the specific binding of dye would not be expected to exceed the specific binding of substrate. If the chromophore could be detected by fluorescence rather than by absorption, the direct determination of the location of an enzyme might be possible.

The most common procedure for the direct cytochemical determination of the location of an enzyme is to apply a colorless substrate that can be converted to a colored product by the specific action of the enzyme. Optical sensitivity is amplified by reactions in which one molecule of enzyme forms a large number of molecules of light-absorbing products. Unfortunately, the locus at which the product reaches a sufficient concentration to be detected need not be, and frequently is not, the site of its formation. If the enzyme is distributed in cytoplasm, the colored product would be formed throughout the cytoplasm; yet the product would not be detected if its solubility in aqueous solution is low. The colored product would be observed only in regions in which it separates from the aqueous phase by precipitation or by solution in a non-aqueous phase. The uncertainty of direct cytochemistry is well illustrated by the following example. It has been assumed that if the respiratory enzymes of bacteria are housed in a cytoplasmic granule analogous to the mitochondrion, the enzymes in the granule should reduce tetrazolium dyes to colored formazans which would accumulate within the granule. Intensely absorbing granules in the cytoplasm are observed following the reduction of tetrazolium salts by a variety of bacteria; these granules were assumed to be identical with dense granules in electron micrographs which are particularly prominent in mycobacteria and in *Micrococcus cryophilus* (10, 11, 12). The interpretation of these granules containing formazan as structural elements of the cell containing the respiratory enzymes is inconsistent with many other observations. The sequence after application of the tetrazolium salt—a large number of small granules near the periphery of the cell followed by the appearance of fewer, larger and more central granules—is inconsistent with the view that the granule is a structural element. Fur-

thermore, an extract of *Bacillus megaterium*, previously stained with triphenyltetrazolium, could be centrifuged to remove all of the color; yet, the colorless supernatant liquid produced additional colored granules on incubation with triphenyltetrazolium chloride and glucose, suggesting that the granules are actually precipitates of insoluble formazan (13). Finally, observation of the same cells by both light and electron microscopy reveals that the stained areas are neither identical in number nor congruent with the electron dense regions which correspond with the familiar metachromatic granules (14).

ANALYTICAL MORPHOLOGY

The lack of sensitivity and specificity of direct cytochemistry are not shared by analytical morphology. If a given structure can be isolated in bulk from a population of cells, the enzymatic composition of that structure can be established by conventional biochemical methods. Analytical morphology requires suitable methods for the release of a given structure from the cell, preferably intact, and purification from other components of the cell as well as adequate criteria of the morphological identity and purity of the final preparation. The most demanding of the several requirements is the method used to open the cell envelope and release intracellular structures.

Disruption.—Most of the physical methods that have been used to disrupt bacteria for the extraction of enzymes—sonic treatment, grinding with abrasive, and various types of ballistic disintegration—result not only in opening the cell but also in the comminution of larger elements of the cell to a mixture of submicroscopic fragments. Not all of the mechanical methods of disruption are as damaging to subcellular structures as grinding or sonic treatment. The cell envelopes of Gram-negative organisms can be broken with a minimum of comminution in the Hughes press (15) or in the French pressure cell (16). These methods are not without effect on the subcellular structures, however. A more sensitive index of the mechanical degradation suffered by the intracellular contents is the degree of polymerization of the DNA released. DNA is usually recovered in the "soluble fraction" after mechanical disintegration; i.e., its sedimentation constant is $\ll 20S$ (17).

The ideal method of disruption for the purposes of analytical morphology is one which acts selectively on the envelope of an intact cell, perhaps by the genesis of a small rent sufficient for the escape of the intracellular contents, but which avoids further disintegration of the parts. One of the most successful methods which fulfills this requirement is lysis by osmotic methods. The cell wall may be hydrolyzed in hypertonic solution to form protoplasts that may be emptied of cytoplasmic contents by subsequent osmotic shock (18). As soon as the membrane is destroyed, no further force exists, and the subcellular fragments escape further mechanical damage. This method has proved particularly useful in isolation of components of the cells of *Bacillus megaterium* and *Micrococcus lysodieticus*, the walls of which are completely

hydrolyzed by lysozyme. The possible damage by osmotic shock to cellular structures other than the envelope may be avoided by opening the protoplast membrane with lipase (19).

Organisms which are not converted to true protoplasts may also be disrupted by osmotic shock if the envelope is weakened sufficiently to permit osmotic lysis in hypotonic solution. Gram-negative bacteria can be osmotically lysed if the envelope is weakened by combined treatment with lysozyme and the removal of divalent cations (20), by growth in the presence of penicillin (21), or by treatment with normal serum (22). Direct osmotic lysis of some bacteria is possible if the intracellular concentration of solutes is sufficiently high; this avoids the possible complications resulting from enzymatic hydrolysis. Halophiles which have been shown to have high internal salt concentrations may be lysed by dilution of the growth medium (23, 24). *Azotobacter agilis*, *Rhodospirillum rubrum*, and *Serratia plymuthica* have been disrupted by osmotic shock after increasing the solute concentration of the cytoplasm with glycerol; unfortunately, this method fails to lyse a variety of other bacteria (25). Disruption by osmotic shock, in contrast with other methods, yields highly polymerized DNA which may be sedimented at low centrifugal force.

In addition to the mechanical disintegration of the subcellular structures, secondary physical destruction may result from the exposure of the contents of the cell to a new environment. The best known and most dramatic example is the degradation of the ribosomes. In media of low ionic strength and low concentrations of Mg^{++} the large ribosomes dissociate to smaller subunits, and the latent ribonuclease contained in the particle becomes active, hydrolyzing the RNA to oligonucleotides with a complete loss of the original structure (26, 27, 28). Obviously, the suspending fluid used for disruption and fractionation should replicate as much as possible the composition of the bacterial cytoplasm, for the preservation of both structure and enzymatic activity. Fortunately, not all of the subcellular structures are as sensitive to small changes in the composition of the suspending fluid as the ribosomes.

Fractionation.—Following the disruption of the bacterial cells in a suitable suspending fluid, the individual subcellular structures may be separated according to their mass and density by successive centrifugation at progressively higher centrifugal forces, sometimes using a density gradient to improve resolution. The extract may be resolved into three fractions by an initial differential centrifugation: (a) structures large enough and with sufficient contrast to be observed with the light microscope may be sedimented at low centrifugal forces such as $10,000 \times g$ for 15 min.; (b) submicroscopic particles which sediment at $100,000 \times g$ for 1 to 2 hr. ($>20S$); and (c) the soluble fraction which contains the substances that remain in solution after the separation of submicroscopic particles. Because of the lack of easily recognizable structures, particularly in extracts prepared by the usual methods of mechanical disruption, the program of differential centrifugation has usually been designed to suit the convenience of the investigator rather than

to yield maximum resolution of the components. This practice is reflected in the nomenclature proposed by Alexander (29) which is based only on the centrifugal force and time of centrifugation used to precipitate the fraction. For example, a fraction obtained as the precipitate by centrifugation at $10,000 \times g$ for 15 min. would be designated as 10p15 in which the prefix designates the mean centrifugal force in $g \times 10^{-3}$ and the suffix the time of centrifugation in minutes. This provisional nomenclature should be retained until the homogeneity of a given fraction is established, and the fraction is identified with a structural element of the cell.

Ideally, the fractionation should be designed for maximum resolution of the structural elements of the cell or for the specific fragments. Examination of each fraction by critical electron microscopy will serve not only to identify the subcellular structure which the fraction contains, but also will aid in the estimation of the purity of the fraction. Electrophoresis and ultracentrifugation are useful in establishing the homogeneity of the fraction and as a means of further resolution of the components. Electrophoresis has proved particularly useful in the resolution of mixtures of ribosomes from the polydisperse fragments of the cell envelope (30), while ultracentrifugation in a density gradient has been used to separate the size classes of ribosomes (31). Finally, one of the most useful methods for establishing the homogeneity of the preparation is the use of immunological techniques. The careful work of Vennes & Gerhardt (32) is an excellent example of the application of immunological methods to determine the purity of subcellular fractions. In this study the isolated capsule, flagella, cytoplasmic membrane, nuclear bodies, and cytoplasmic granules of *Bacillus megaterium* were examined for serological cross-reaction by complement fixation. The only significant cross-reactions were between capsular polysaccharide and cell wall, which was interpreted as evidence for the presence of the same polysaccharide in both capsule and wall, and between cytoplasmic membrane and flagella, suggesting that the cytoplasmic membrane contains portions of the flagella.

RESPIRATORY ENZYMES

The bulk of the respiratory enzymes of bacteria that are disrupted by grinding with abrasive or by sonic treatment are recovered in the centrifugal fraction which contains submicroscopic particles. The enzymatic properties and chemical composition of this fraction have been thoroughly reviewed by Alexander (29) and by Mitchell (1). A partial list of the enzymatic activities of the particulate portion of some representative bacteria are given in Table I. The submicroscopic particles exhibit the same absorption bands of cytochromes as the whole cell. Only small amounts of cytochromes are found in the other centrifugal fractions. The particles also contain flavins which contribute to the decrease in light absorption at $450 \text{ m}\mu$ on reduction. The particles contain the complete system for the oxidation of reduced diphosphopyridine nucleotide (DPNH) and reduced triphosphopyridine nucleotide (TPNH), and for the coupled phosphorylation of adenosine diphosphate.

TABLE I
ENZYMATIC ACTIVITY OF THE PARTICULATE FRACTIONS

Organism	Enzymes	References
<i>Azotobacter agilis</i>	Hydrogenase, lactic, malic, and succinic dehydrogenases, DPNH and TPNH oxidases	(30, 33 to 36)
<i>Pseudomonas fluorescens</i>	Glucose, gluconate, malic, mandelic, <i>p</i> -hydroxymandelic, and succinic dehydrogenases, DPNH and TPNH oxidases and nicotinic hydroxylase	(37, 38)
<i>Escherichia coli</i>	Hydrogenase, α -glycerophosphoric, formic, lactic, and succinic dehydrogenases and DPNH oxidase	(39, 40)
<i>Proteus vulgaris</i>	Hydrogenase, formic, lactic, and succinic dehydrogenases and DPNH oxidase	(41)
<i>Serratia marcescens</i>	α -glycerophosphoric, α -ketoglutaric, formic, lactic, malic, and succinic dehydrogenases	(42)
<i>Acetobacter suboxydans</i>	Polyol dehydrogenase(s) for glycerol, erythritol, mannitol, and sorbitol, alcohol dehydrogenase	(43)
<i>Gluconacetobacter liquefaciens</i>	Polyol dehydrogenase(s), glucose, gluconate, and 2-ketogluconate dehydrogenases, glycerokinase, lactic, and succinic dehydrogenases, DPNH oxidase, and catalase	(22)
<i>Mycobacterium avium</i>	Succinic and malic dehydrogenases, DPNH and TPNH oxidases	(44, 45, 46)
<i>Micrococcus aureus</i>	Succinic, malic, lactic, formic, and α -glycerophosphoric dehydrogenases, malic enzyme, acid phosphatase, and succinate activating enzyme	(47)

The range of substrates oxidized by washed submicroscopic particles is quite limited. Most of the dehydrogenases which reduce pyridine nucleotides are soluble enzymes; thus, the respiration of the substrates requires the co-operative action of the soluble dehydrogenase which produces reduced pyridine nucleotides, DPNH or TPNH, and the particulate complex of enzymes responsible for reoxidation of the pyridine nucleotide and coupled phos-

phorylation. The submicroscopic particles contain the dehydrogenases for succinate, formate, and α -hydroxy acids (lactate, malate, mandelate). Hydrogenase is usually found in the submicroscopic particles present in both autotrophic and heterotrophic bacteria which form this enzyme (48). Polyol dehydrogenase and alcohol dehydrogenase are uniquely present in the particles from *Acetobacter*.

A few of the enzymatic activities are found both in particles and as soluble enzymes. For example, the submicroscopic particles from *Acetobacter suboxydans* contain a glucose dehydrogenase while the soluble fraction contains a DPN-linked glucose dehydrogenase (49). Also, the polyol dehydrogenase, which is particulate, has the substrate specificity of the Bertrand-Hudson enzyme, and the soluble polyol dehydrogenase(s) has both a different structural specificity for the substrate and a more alkaline pH-optimum. Malic dehydrogenase of *Azotobacter vinelandii* (34) and *Bacillus megaterium* (50) is found in both the submicroscopic particles and the soluble fractions suggesting the presence of two distinct enzymes for the oxidation of malate; in *Mycobacterium avium* this enzyme is restricted to the particles, and in *M. tuberculosis* it is entirely soluble (51, 52). Succinic dehydrogenase is exclusively in the particles; however, most of the enzymatic activity is lost by washing the particles and is restored by mixing the particles and soluble fraction. Although this result might be interpreted as evidence that one component of the system is soluble, it actually results from inhibition of the succinic dehydrogenase of the particles by traces of oxalacetate which accumulate. The soluble fraction, which contains the oxalacetic decarboxylase, relieves this inhibition (35, 45).

The chemical composition of the particles obtained from a variety of bacteria is quite similar (29). The particles are rich in ribonucleic acid and phospholipid; as much as 85 per cent of both the RNA and phospholipid of the cell may be recovered in this fraction. Analytical ultracentrifugation reveals discrete components in the sedimentation range of 20S to 50S; the pattern obtained is largely independent of the method of disruption (17). Electron microscopy of the submicroscopic particles shows two types of particles: one is a regular spherical structure dense to electrons and approximately 200 Å in diameter; the other is flattened, more irregular in size and shape, less dense to electrons, the larger elements of which sometimes take the form of discs (30, 44).

In addition to the mixture of at least two types of particles in electron micrographs, the heterogeneity of the submicroscopic particles has been established by a variety of other means: (a) The rates of release during sonic treatment of the substances found in the submicroscopic particles are not identical. Soluble enzymes and the particles containing RNA are released at the same rate as the disruption of the cells, which is compatible with the postulate that the particles containing the RNA exist as such in the cytoplasm. The respiratory enzymes and phospholipid are released in submicroscopic form at a rate lower than the rate of disruption of the cells; thus, the

submicroscopic particles which contain these enzymes must be derived by the comminution of a larger structure (53, 54). (b) The submicroscopic particles can be resolved into two or more classes by zone electrophoresis (30) or partially resolved by iterated differential centrifugation (44). The particles containing the RNA, and which are responsible for the discrete boundaries observed in the ultracentrifuge, can be identified with the regular spherical particles observed in electron micrographs of the particle fraction and in the cytoplasm of the cell in thin sections. These particles, which have been termed ribosomes (55) do not contain the respiratory enzymes (30). The parent structure, from which the submicroscopic particles containing the respiratory enzymes and phospholipid are derived, may be isolated by methods of disruption less drastic than the conventional grinding or sonic treatment.

The membrane has been established as the locus of the respiratory enzymes of *Bacillus megaterium* by the elegant work of Weibull. The membrane, which may be obtained essentially intact by conversion of the cells to protoplasts with lysozyme followed by the osmotic lysis of the protoplast, was shown to contain the bulk of the cytochromes. The prediction by Stanier (56) that the membrane was the parent structure from which the submicroscopic particles containing the respiratory enzymes were derived, has been confirmed by finding of succinic, malic, lactic, and α -ketoglutaric dehydrogenases and DPNH oxidase in the isolated membranes of *B. megaterium* (50, 57). Although the isolated membranes enclose a few granules visible by light or electron microscopy, it appears that the membrane and not the granules is the locus of the enzymes, since the enzymatic activity is not dependent on the degree of contamination of the membranes by granules.

Militzer and his co-workers have obtained a large structure containing the respiratory enzymes following the treatment of *Bacillus stearothermophilus* with lysozyme (58, 59, 60). Although it has been implied that this fraction constitutes some intracellular structure (61, 62), it is clear from phase photomicrographs that this fraction consists of whole or lysed protoplasts (56). This interpretation is consistent with the large number of enzymes found in the structure. After more thorough washing of the isolated structure, it was found to comprise 20 per cent of the dry weight of the cells and to contain most of the cytochromes, lipid, organic iron, and ATPase (63). Electron micrographs of this material show a membrane surrounding several granules 850 A in diameter; the electron micrographs are almost identical to those of the lysed protoplasts of *Bacillus megaterium* M. (64). Georgi, Militzer & Decker (63) apparently overlook the possibility that the enzymatic activities are in the membrane rather than associated with the granules. The evidence is compatible with the hypothesis that the membrane is the locus of the respiratory enzymes; however, the isolation of the granules from the membranes and the determination of their chemical and enzymatic composition will be necessary to convince the remaining critics.

Mitchell & Moyle have obtained an almost intact membrane of *Staphylococcus* (*Micrococcus*) *aureus* by partial autolysis of the cell wall followed by

osmotic shock (65). The isolated membrane, which is stated to be free of granules, is readily converted to submicroscopic particles identical with the particles produced by mechanical disintegration of the cell. The enzymes recovered in the membrane of *S. aureus* were the same as those found in the submicroscopic particles.

The envelope of Gram-negative bacteria has not been successfully separated into layers which can be defined as wall and membrane. Electron micrographs of thin sections of the Gram-negative bacteria show a multi-layered envelope. The portion of the envelope, which has been termed the cell wall, appears as a laminate of a more transparent zone between two electron-dense layers; a third dense layer which sometimes adheres to the cytoplasm and sometimes adheres to the wall is assumed to be the cell membrane (66, 67). Unfortunately, no method is yet available for the separate isolation of individual layers of the envelope. The structures isolated from enterics were defined as cell walls by Salton because they were isolated by the same methods used to recover the cell walls of the bacilli and micrococci; however, walls isolated from the Gram-negatives have high contents of lipid and complete proteins (68), more strongly resembling the chemical composition of the membrane (69, 70) rather than the cell wall of the Gram-positive organisms. The conversion of cells to spheroplasts by treatment with lysozyme does not ensure that a distinct layer has been removed or even that the mechanical strength of the envelope resides exclusively in a layer (wall) separate from that which controls diffusion of low molecular weight solutes (membrane).

The envelope of the Gram-negative bacteria has been isolated by a variety of methods and, like the membrane of the Gram-positive bacteria, has been demonstrated to contain the respiratory enzymes. The empty envelope of *Azotobacter vinelandii* was isolated, following brief sonic treatment, by differential centrifugation to separate it from residual intact cells. The envelope was found to contain cytochromes, hydrogenase, succinic and malic dehydrogenases, and DPNH oxidase (30). Although this method results in only small yields of the envelope, the specific activities of the enzymes found in the envelope were as high or higher than the specific activities of the same enzymes isolated as submicroscopic particles, which is compatible with the conclusion that the membrane is the parent structure from which the submicroscopic particles are derived by comminution. Jose & Wilson (71) prepared spheroplasts of *A. vinelandii* by several means and found the hydrogenase in the particulate fraction presumably derived from the membrane. Robrish & Marr (25) recovered the empty envelopes of *A. vinelandii* in high yield following direct osmotic shock with glycerol. The centrifugal fraction containing the envelopes also contained 85 to 90 per cent of the cytochromes, hydrogenase, and DPNH oxidase. Although the envelopes obtained by direct osmotic shock are not entirely homogeneous in electron density, they are quite flat and contain no granules of the dimensions of the chondrioids found in thin sections of some bacteria. Hunt, Rodgers & Hughes (15) obtained the

envelope of *Pseudomonas fluorescens* by disruption of the cells in a Hughes press, which apparently does not appreciably comminute the envelope. The low-speed centrifugal fraction which contained the emptied envelopes also contained 90 to 95 per cent of the nicotinic acid hydroxylase with a specific activity over three times that of the whole extract. The envelope also contained succinic dehydrogenase, DPNH and TPNH oxidases, and the associated electron-transport system. Stouthamer (22) has obtained the envelope of *Gluconacetobacter liquefaciens* by treatment of cells with normal serum in hypotonic media. The resulting ghosts contained the same enzymes as did the small particle fraction from mechanically disrupted cells.

There remains little doubt that the parent structure for the submicroscopic particles containing the respiratory enzymes is the cell membrane in bacilli and micrococci and is some portion of the envelope of the Gram-negative organisms. The respiratory enzymes are not in the 200 A particles in the cytoplasm or in the chondrioids observed in thin sections.

A more precise description of the location of the respiratory enzymes will require further analysis of the structure of the envelope. One approach to this problem is the disintegration of the isolated envelope to small particles followed by resolution of the fragments by physical means. The sonic comminution of the envelope of *A. vinelandii* releases both cytochrome and rhamnose at rates identical with the loss of dry weight, which implies that the cytochromes are an integral part of the envelope and are not contained in a structure loosely associated with the envelope. The fragments obtained have been separated by electrophoresis into classes which differ in chemical composition, suggesting that the envelope is heterogeneous.

The similarity in both chemical and enzymatic composition between the membrane (or envelope) of bacteria and the mitochondrial membrane is striking. For those who have sought homologies in the localization of respiratory enzymes in bacterial cells and in cells of higher organisms, the most appropriate choice of a structure homologous with the mitochondrion is the entire bacterial cell rather than the deposits of stain which accumulate internally. The bacterium is unique among microorganisms since mitochondria of typical morphology have been demonstrated in yeast, filamentous fungi, and protozoa.

RIBOSOMES AND PROTEIN SYNTHESIS

The submicroscopic particles of ribonucleoprotein, which have come to be known as ribosomes, were first discovered in bacteria by Schachman, Pardee & Stanier (17). These particles are the principal if not the exclusive components of bacterial extracts responsible for boundaries in the ultracentrifuge with sedimentation constants in the range of 20 to 100S. Much of the earlier work on the composition of the ribosomes was confused by gross contamination of the centrifugal fraction containing the ribosomes with fragments of the envelope. Ribosomes have been purified from the fragments of the envelope by zone electrophoresis (30) and by differential or density

gradient centrifugation (31, 44). Of the two methods, electrophoretic separation offers the highest resolution because of the large difference in the surface charge of the two types of particles. Centrifugation is less reliable; the wide range of size of the fragments of the envelope distributes these fragments among the centrifugal fractions. Analytical centrifugation is also inadequate as a criterion of purity of the ribosomes from contamination by fragments of the envelope which are too polydisperse to form boundaries.

Ribosomes are identical in appearance with the electron-dense particles that are found distributed throughout the cytoplasm in thin sections of bacteria (5). This location in the cytoplasm is confirmed by the kinetics of release of the RNA during sonic treatment and by the similar patterns obtained by analytical ultracentrifugation of extracts prepared by different means of disruption. Both methods suggest that the ribosome is an extant structure, not a fragment produced by the disruption of the cell.

Size classes of ribosomes corresponding to approximately 20S, 30S, 50S, 70S, and 85 to 100S have been described for *Escherichia coli* (16, 72). The total ribosome content is proportional to the growth rate. If growth stops from the exhaustion of glucose, almost all of the ribosomes are quickly converted to 100S. When growth resumes, the smaller ribosomes are formed (73). The significance of the smaller ribosomes is confused by the spontaneous conversion of the larger ribosomes to smaller ones as the magnesium concentration is lowered. For example, the 70S ribosome dissociates into 50S and 30S components below 0.002 *M* Mg. However, the smaller ribosomes most rapidly incorporate P^{32} -orthophosphate while the larger ribosomes most rapidly acquire S^{35} from inorganic sulfate. These results suggest that the smaller size classes are not simply an artifact from dissociation of the larger ribosomes but are possible precursors in the biosynthesis of the larger ribosomes (73).

Ribosomes are composed of approximately 60 per cent nucleic acid and 40 per cent protein or about two amino acid residues per nucleotide (16, 72). After high speed centrifugation sufficient to precipitate the particles of 20S or more (2 hr. at 100,000 \times g), 80 to 90 per cent of the RNA is sedimented; the remaining 10 to 20 per cent is the soluble RNA (72). The reports of large amounts of soluble RNA prior to the establishment of the conditions for stability of the ribosomes reflect a partial activation of the latent ribonuclease and subsequent hydrolysis of the ribosomal RNA (29).

Only three enzymes have been found to be located principally in the ribosomes. A latent ribonuclease which can be activated by urea or versene was found in the ribosomes of *Escherichia coli* (74); active ribonuclease also develops in the ribosomes of *A. vinelandii* stored in phosphate buffer (30). Only 11 per cent of the total ribonuclease of *E. coli* is not sedimented with ribosomes; however, this, like the sedimented enzyme, is latent and may represent unsedimented ribosomes (73). Thus, most, if not all of the ribonuclease of *E. coli*, is contained in the ribosomes. Recently, Elson (75) has reported a latent deoxyribonuclease in the ribosomes of *E. coli* which is sepa-

rable from the ribonuclease by electrophoresis. Leucine amino peptidase is also found in the ribosomes of *E. coli* but is present in varying but substantial amounts in the soluble fraction as well (73). Leucine amino peptidase found in the ribosomes is not latent. The most convincing evidence that leucine amino peptidase is actually a constituent of the ribosomes is that the specific activity of this enzyme remains constant in ribosomes fractioned by density-gradient centrifugation.

The long-standing circumstantial evidence for the involvement of nucleic acid in the synthesis of protein implicates the ribosomes, which contain most of the RNA, as a site of protein synthesis. The complete process of protein synthesis from the activation of amino acids to the final assembly into protein has been approached by two different methods. One method is the study of the synthesis of protein by disrupted cells or subcellular structures by measuring the incorporation of radioactive amino acids. The second method is the measurement of the kinetics of incorporation of tracer amino acids (or radi sulfate) by intact cells into subcellular fractions obtained subsequent to incorporation. The two methods applied to bacteria have given apparently dissimilar results.

The subcellular fractions of bacteria most active in the incorporation of amino acids *in vitro* have contained cell envelopes or fragments of cell envelopes [reviewed by Roberts, McQuillen & Roberts (76)]. The results of many of these experiments are difficult to interpret both because of the uncertainty of the products containing the labeled amino acid and because of the lack of morphological definition of the fraction studied. For example, single amino acids are incorporated by the envelopes from *Staphylococcus aureus* largely either into the cell wall peptide or into soluble RNA-amino acid complexes rather than into complete proteins (77, 78). Further resolution of the active subcellular systems is complicated by the numerous reactions required in the conversion of amino acids into protein, not all of which would be expected to reside in the same structure. Despite the difficulties in interpretation of the results of these experiments, the subcellular fractions containing the envelopes of the cell are clearly the most active in converting amino acids into products which resemble proteins (79, 80).

The measurement of the incorporation of labeled amino acids by intact cells of *E. coli* into subcellular fractions implicates both the soluble RNA and the ribosomes in the synthesis of the soluble proteins of the cell, and is consistent with the findings with animal cells. Lacks & Gros (81) have found that the soluble RNA incorporates labeled amino acids more rapidly than either the ribosomes or the soluble protein, and that the rate of formation of the complex between soluble RNA and amino acid is adequate to account for the rate of synthesis of protein. Also, after the accumulation of a pool of the complex in the presence of chloramphenicol which inhibits the synthesis of protein, the label is transferred to soluble protein when the inhibition is relieved.

McQuillen, Roberts & Britten (82) have found that the rate of labeling

of the ribosomes of *E. coli* by cultures growing in the presence of S^{35} -sulfate and the rate of transfer of this label from the ribosomes to the soluble protein, is adequate to account for the rate of synthesis of soluble protein. The transient labeling of the ribosomes saturates after about 5 sec. Only the large ribosomes (70S or larger) became appreciably radioactive in short exposures to radiosulfate. The identity of the radioactive product and the conditions for its release from the particles *in vitro* have not yet been established.

Although it is tempting to accept the labeling of the larger ribosomes as evidence for their participation in the synthesis of soluble proteins, the data do not exclude the possibility that the label is associated with fragments of the envelope which are produced by the disruption in the French press and which could heavily contaminate the centrifugal fractions containing the larger ribosomes. The low specific activity of ribosomes recovered by lysis with lysozyme was interpreted as evidence that the ribosomes free in the cytoplasm were less active in the synthesis of protein while those associated with the envelope were more active. However, the result may be explained equally well by assuming that lysis with lysozyme results in less fragmentation of the envelope than lysis in the French pressure cell. Electrophoretic separation of the ribosomes from contaminating fragments of the envelope would relieve this doubt.

If the ribosome is assumed to be the site of assembly of the amino acids from "adapter-amino acid complexes" into the various proteins which are ultimately released into the cytoplasm, it should be possible to demonstrate the assembled enzyme on or in the ribosomes. The discovery of a latent form of β -galactosidase in ribosomes from *E. coli* which can be activated by reaction with antiserum to β -galactosidase most closely fulfills this requirement (73).

PHOTOSYNTHETIC PIGMENTS

Photosynthetic bacteria and blue-green algae lack the chloroplasts typical of all other photosynthetic forms. The pigments of the photosynthetic bacteria appear by light microscopy to be distributed throughout the cell rather than concentrated in intracellular structures; however, after mechanical disruption of photosynthetic bacteria, the pigments can be recovered, highly concentrated, in particles which sediment in 1 hr. at $25,000\times g$. The particles containing photosynthetic pigments were independently discovered by Schachman, Pardee & Stanier (17) who referred to the granules as chromatophores, and by Thomas (83) who called them grana. The term chromatophore has come into general use, because it does not suggest a relationship between the particles from photosynthetic bacteria and the grana of chloroplasts. Both electron microscopy and analytical ultracentrifugation indicate that the chromatophore is a particle of discrete dimensions. The chromatophores of *Rhodospirillum rubrum*, air dried from a mist, appear as discs with a mean diameter of 900 Å and a range of 500 to 1600 Å (84). Chromatophores of *Rhodospirillum* have a sedimentation constant of approximately 200S (17).

Chromatophores isolated from *Chromatium* are somewhat smaller but otherwise similar to those obtained from *Rhodospirillum* (85). On disruption, *Chlorobium* does not yield typical chromatophores; the carotenoid pigments sediment at relatively low centrifugal force but the chlorophyll is sedimented only after prolonged centrifugation (86).

Vatter & Wolfe (87) found vesicles of the same dimensions as isolated chromatophores in thin sections of *Rhodospirillum*, *Rhodopseudomonas*, and *Chromatium*. Light-grown *Rhodospirillum rubrum* contained numerous ellipsoids with a major axis of 500 Å, the interior of which was transparent to electrons while the cortex was more opaque. Comparable structures were absent from dark-grown cells. Earlier observations of thin sections of *Rhodospirillum* had failed to show chromatophores but rather revealed paired or multiple lamellae (88, 89). The careful study by Hickman & Frenkel (90) has demonstrated that the age of the culture of *Rhodospirillum* determines the appearance of the thin section. Cells from cultures 12 hr. old or less show neither vesicles nor multiple lamellae. The photosynthetic pigments from young cultures are recovered in particles smaller than chromatophores. After 18 to 24 hr., structures of the same dimensions as the chromatophores appear in the cytoplasm. Initially, the interior of the structure is relatively opaque to electrons, becoming more transparent and comparable to the appearance in the sections photographed by Vatter & Wolfe with increasing age of the culture. The chromatophores persist for about 10 days. Cells in older cultures do not contain chromatophores but rather show the lamellae. The lamellae appear either as independent structures in the cytoplasm or appear aligned parallel with the cell wall. It is by no means certain that the laminated structures house the photosynthetic pigments. Similar laminated structures are found in non-photosynthetic microorganisms, particularly mycobacteria and streptomycetes (91). The changes in internal structure with the age of the culture of *Rhodospirillum* provide an explanation for the lack of chromatophores in *Chlorobium*, which resembles cells from young cultures of *Rhodospirillum* both in the lack of internal structures of dimensions corresponding with chromatophores and in the failure of workers to isolate chromatophores from extracts.

Results of indirect methods of determination of the location of the photosynthetic pigments do not agree with the picture of independent chromatophores distributed throughout the cytoplasm. The photosynthetic pigments of *Rhodospirillum* and *Chromatium* are not released by sonic treatment as free chromatophores at the same rate that the cells are destroyed and the soluble components of the cytoplasm released (92, 93). The pigments sediment with the cell envelope and additional sonic treatment is required for the liberation of the chromatophores. The osmotic lysis of spheroplasts of *Rhodospirillum* prepared by lysozyme and versene (94) or by lysozyme and polymyxin (95) also fails to release the chromatophores even though much larger structures, the particles of poly- β -hydroxybutyrate, are released by this treatment. Again, the photosynthetic pigments sediment with the cell

envelope. It is difficult to reconcile these results with the appearance of thin sections in which apparently independent structures of the same size as chromatophores are prominent in the cytoplasm. It is possible but rather improbable that chromatophore-like vesicles were absent from the cytoplasm of the cells used in all of these experiments and that the pigments were contained in the large laminated structures characteristic of old cells. It is more reasonable to assume that the chromatophores are part of some larger structure—either a separate internal network or the inner surface of the cell envelope itself.

The most detailed study of the chemical composition has been the analysis of chromatophores from *Chromatium* by Newton & Newton (93). The major constituents of chromatophores are protein, phospholipid, and polysaccharide; the molar ratio of chlorophyll:protein:carotenoid is 2:2:1. The chromatophores contain smaller amounts of cytochrome, flavin and pyridine nucleotides, and relatively large amounts of non-heme ferrous iron. The phospholipid contains equimolar amounts of ethanolamine, glycerol, and phosphate. The polysaccharide of the chromatophores is similar in composition to the polysaccharide that can be washed from the cells; both polysaccharides yield only glucose on hydrolysis (96). This component is probably responsible for the strong serological cross reaction observed between the chromatophores and the cell surface. Antisera prepared with chromatophores as the immunizing antigen agglutinate intact cells, and the adsorption of antichromatophore serum with an excess of cells removes approximately half of the precipitating antibody for chromatophores. Either the chromatophores contain a polysaccharide in common with the cell envelope or, perhaps, are attached to fragments of the envelope itself.

Sonic treatment of the chromatophores of *Chromatium* produces particles which do not sediment at $25,000 \times g$ for 1 hr. but sediment at $100,000 \times g$ in 1.5 hr. These smaller particles are almost free of the polysaccharide component of the parent chromatophores and show a twofold increase in the ratio of photosynthetic pigments to protein (93). Sonic treatment of the chromatophores of *Rhodospirillum* yields particles 200 to 400 A in diameter or about one-third the linear dimensions of the parent chromatophores (84). The smaller particles obtained by sonic comminution of chromatophores resemble in size particles which contain the photosynthetic pigments in cells from young cultures of *Rhodospirillum*. The small particle, thus, may be a natural unit rather than a random fragment of the chromatophore.

The enzymatic activities of chromatophores has been recently reviewed by Frenkel (97). As one might anticipate from their content of chlorophyll and carotenoids, the chromatophores house the photosynthetic machinery. Chromatophores catalyze cyclic photophosphorylation of ADP to ATP comparable to that found in chloroplasts of plants without added reducing agents (98, 99, 100). Well-washed chromatophores require the addition of catalytic amounts of succinate for maximum rates of photophosphorylation, the function of which may be to prevent photooxidation by traces of oxygen

which inactivates the system. The cyclic photophosphorylation can provide the ATP requirement for photosynthesis. Indirect spectrophotometric evidence suggests that the cytochromes in the chromatophores transport electrons during photophosphorylation. In addition to the cyclic phosphorylation, the chromatophores catalyze the photochemical reduction of DPN by reduced flavin, presumably by an interruption of the cyclic system of electron transport in photophosphorylation (100). A reaction of this type can provide the reduced pyridine nucleotide that is required in addition to ATP for photosynthesis. Chromatophores differ from chloroplasts in that chloroplasts contain the enzymes responsible for the photosynthetic fixation of CO_2 while the chromatophores do not (101). The enzymes responsible for the fixation of CO_2 are recovered in the soluble fraction in extracts of the photosynthetic bacteria.

ENDOSPORES

The investigation of the enzymatic content of endospores has been possible only since the development of methods of purifying spores from contamination by vegetative cells and debris from lysed cells and sporangia. The enzymes characteristic of the resting spores of aerobic bacilli have been reviewed by Murrell (102) and by Halvorson & Church (103). Only a few studies have been made of the location of these enzymes in the spore, and most of the studies have been made with spores of *Bacillus cereus*.

Some of the enzymatic activities such as alanine racemase, ribosidase, heat-stable catalase, and adenosine deaminase are demonstrable in intact resting spores while others, alkaline pyrophosphatase, heat-labile catalase, and alanine deaminase are cryptic. One possible conclusion is that the cryptic enzymes are inside the permeability barrier while the active enzymes are outside the barrier to diffusion of small molecules and, thus, have access to substrates in the medium. Black, MacDonald & Gerhardt (104) have demonstrated that only 40 per cent of the volume of spores of *B. cereus* are freely permeable to low molecular weight solutes. The permeable volume corresponds roughly to the volume of the exosporium. Either the exosporium or the spore coat is a likely locus for the active enzymes.

More conclusive evidence for the location of certain spore enzymes in the exosporium is provided by selective removal of the exosporium by sonic treatment (105). The spores of *B. cereus* are broken by sonic treatment in two steps: the first interaction removes the exosporium, which apparently prevents the disruption of the spore body, and the second interaction destroys the naked spore. The rate of release of alanine racemase and adenosine deaminase from the spore correlates with the rate of stripping of the exosporium, which suggests that the racemase and deaminase are in the exosporium. Ribosidase is released only after disruption of the spore body and, thus, is not in the exosporium. Spore coats isolated after ballistic disintegration of spores of *B. cereus* have a high specific content of ribosidase. The location of the ribosidase in the spore coat, outside the permeability barrier, would ex-

plain the activity of ribosidase in the intact spore. Recently, O'Conner & Halvorson (106) found that the kinetics of sonic release of one of the cryptic enzymes, alanine deaminase, agree with the location of this enzyme inside the spore body rather than in the exosporium.

The location of enzymes in the spore is apparently related to the mechanism of the resistance of these enzymes to heat. Two hypotheses are usually advanced to explain the resistance of the enzymes of endospores to heat: (a) the enzymes are individually inherently heat-stable or (b) the enzymes are not inherently heat-stable but are generally protected by some structural feature of the spore. The cryptic enzymes, pyrophosphatase and heat-labile catalase, which are located inside the spore body, are stable to heat in the intact spore but are quite sensitive to heating after extraction from the spore (102, 107). Thus, the resistance to heat of these two enzymes and probably other enzymes inside the spore body depends upon the integrity of the spore, possibly a low water activity inside the spore. The racemase and deaminase, which are located in the exosporium, are resistant to heating after release from the spore (105). Ribosidase and *p*-phenylenediamine oxidase, which are located in the spore coat, are also resistant to heat after the spore is destroyed (102, 108).

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THE ARTHROPOD-BORNE VIRUSES OF MAN AND OTHER ANIMALS^{1,2}

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The arthropod-borne viruses of man and animals now number more than fifty. They are found on all the inhabited continents of the world and in many of the insular areas. During the past six years many of these viruses have been systematically investigated in respect to their vector-host relationships. It is the intent of this review to cover the ecological aspects which have been studied during this period. Important advances have also been made in the classification of these agents, and in the field of laboratory diagnosis. It would be impossible to cover these phases in the allotted space. Because of the practical importance of classification of these viruses to the understanding of their geographic distribution and interrelationships, the reader is referred to a summary of their grouping by Casals (1), and to the techniques employed for this grouping by Clarke & Casals (2).

GROUP A VIRUSES

Western equine encephalitis (WEE).—Aside from yellow fever, WEE has received more intensive investigation than any of the other arthropod-borne viruses. The information gathered in the years prior to those reviewed here left little doubt that the principal vector of this virus is *Culex tarsalis*; that birds are the primary hosts, and that man and horses can be considered "accidental" hosts for the virus.

Much of the investigative work on WEE in recent years has been directed toward elucidating the mechanisms whereby the virus survives the winter months. Cockburn *et al.* (3) reported the chronology of WEE isolations from mosquitoes and birds in an endemic area of Colorado over a four-year period. The agent was isolated each year from both mosquitoes and birds. The earliest isolation in any year from birds was in June, while the latest isolation was in September. The earliest and latest isolations from mosquitoes collected in the study area occurred in the same months. The bulk of the 32 mosquito isolations was from *C. tarsalis*, but one isolant was obtained from the tick *Dermacentor americanus* during July, 1950. As part of this study Blackmore & Winn (4) reported the extremely interesting isolation of WEE virus

¹ The survey of the literature pertaining to this review was concluded in December, 1959.

² The following abbreviations will be used: CTF (Colorado tick fever); EEE (Eastern equine encephalitis); JBE (Japanese B encephalitis); MVE (Murray Valley encephalitis); SLE (St. Louis encephalitis); VEE (Venezuelan equine encephalitis); WEE (Western equine encephalitis); WN (West Nile fever); YF (Yellow Fever).

from one of 14 lots of hibernating *C. tarsalis* collected in an abandoned mine in December, 1953, in the foothills of the Rocky Mountains lying outside of the principal study area. In warmer climates, such as in California, the virus is active over a larger portion of the year. Longshore *et al.* (5) in the course of epidemiologic observations in California over an eight-year period, found the earliest human cases of WEE to appear in June and the latest in October. There was a tendency for a northward movement of the disease in man through the central valley of California, this they interpreted as due to the sequential development of optimum climate for mosquito activity. Hess & Holden (6) state that resident (rather than migratory) avian hosts or arthropod vectors are the more likely winter reservoirs of WEE virus. Reeves, Bellamy & Scrivani (7) have isolated WEE from *C. tarsalis* during all months of the year except December in Kern County, California. That these were not necessarily truly hibernating mosquitoes is indicated by the observation that virus was isolated only at times when blood-engorged mosquitoes were also found. Ninety per cent of the engorged *C. tarsalis* found from January to May contained nucleated red blood cells, indicating the probability that they had fed on avian hosts. The January-to-April isolants of WEE differed from the usual summer ones in that they displayed a remarkably low pathogenicity for mice. Bellamy *et al.* (8) experimentally infected *C. tarsalis* with WEE and, after a 10-to 13-day holding period at 75° to 84°F., placed them in a constant temperature cabinet at 55°F. Virus persisted in these mosquitoes for 41 days. When infected mosquitoes were placed in an unheated cellar in Kern County, California, during the winter, the virus survived in them for 113 days, and was transmitted to chickens after 97 and 109 days. These authors hypothesized a two-step mechanism of winter virus survival: first, mosquitoes infected in the late fall may survive for three months and feed on susceptible birds in January, and secondly, "new" mosquitoes become infected by feeding on these birds, and emerge in the spring to start a new season of virus activity. The possibility that latently infected birds may serve as reservoirs for the virus is presented by Reeves and associates (9) who were able to isolate it from 7 of 284 experimentally infected birds between 55 and 245 days after inoculation; on two occasions, from the blood. Attempts by Reeves (10) to produce, experimentally, long-term infections with WEE in two mite species, *Bdellonyssus sylviarum* and *Dermanyssus americanus*, were unsuccessful. Sulkin *et al.* (11) likewise failed to effect transmission of WEE to chickens with *Dermanyssus gallinae*. Chamberlain & Sikes (12), working with *D. gallinae*, *B. sylviarum*, and *B. bursa*, found these mites to retain WEE virus for only two to five days, generally. In one instance, the virus was found in the excrement on the sixth day, and *D. gallinae* transmitted WEE to a chick on the thirteenth day. It was the consensus of opinion of these last three groups of workers that, in view of their low susceptibility, any role these particular species of mites play in the transmission of infection must be minor. Winn & Bennington (13) failed to isolate WEE virus from 489 nasal mites of the genus *Ptilonyssus* and 245

of the genus *Speleognathus* collected from 44 English sparrows 20 days after experimental inoculation.

The role of mammals has been thought to be minor in the cycle of WEE virus maintenance because earlier serological surveys showed a much lower incidence of antibody in mammals than in birds. Cockburn, Sooter & Langmuir (3), during their study in Colorado, found 6 per cent of small wild mammals (mostly jackrabbits) and only one of 115 domestic rabbits to have WEE neutralizing antibody. Lennette and colleagues (14) have isolated WEE virus on three occasions from tree squirrels (*Sciurus griseus*) and on two occasions from ground squirrels (*Citellus beecheyi*), in all cases from animals suspected of being rabid. In view of the apparent high susceptibility of these animals to clinical disease the authors suggest that squirrels probably do not serve as true reservoirs of the virus. Kissling (15) has reported that experimentally inoculated horses have fleeting viremias of very low titers. In general, however, mammals have not been experimentally investigated very thoroughly in regard to their potential for serving as hosts for WEE virus. Dow *et al.* (16), using mosquito traps designed to eliminate as many variables as possible, found that although *C. tarsalis* was attracted to a wide variety of avian and mammalian species, it was more often attracted to the former.

The distribution of the virus in North America was believed to be limited to the distribution of *C. tarsalis* until Kissling *et al.* (17) reported its isolation from three different species of wild birds, and from the mosquitoes *Culiseta melanura* and *Aedes infirmatus*, in Louisiana. Chamberlain (19) has reported isolations of WEE from *C. melanura* in New Jersey and from engorged *C. pipiens-quinquefasciatus* in North Carolina. Holden (18) isolated the virus from naturally infected English sparrows in New Jersey, and Stamm (20) has made additional isolations from the blood of wild birds collected in that state. Feemster (21) reports the presence of neutralizing antibody in wild birds in Massachusetts. Chamberlain (19) suggests that the ecology of WEE virus in the eastern United States is similar to that of EEE virus. Kissling and co-workers (22) have shown that an eastern isolate of WEE can easily infect several small bird species indigenous to the southeastern United States, the titer of virus in the blood attaining $10^{4.0}$ to $10^{6.0}$ LD₅₀.³ The viremia generally lasted for four days, and it was possible to effect bird-to-bird transmission of the virus with *Aedes triseriatus* and *A. aegypti*. Chamberlain *et al.* (23) showed that the extrinsic incubation period in these *Aedes* species was five days after feeding on blood containing the maximum virus titer. Optimum transmission was reached in 13 days by *A. triseriatus* and somewhat sooner by *A. aegypti*. Barnett (24) determined the mean extrinsic incubation period for WEE in *C. tarsalis* to be approximately eight days with a minimum of four days. Chamberlain & Sudia (25) determined that

³ Throughout this review LD₅₀ titers refer to the quantity contained in a mouse intracerebral dose, i.e., 0.03 to 0.04 ml.

the infection threshold for *Culex tarsalis*, i.e., the amount of virus necessary to initiate infection, was only 10 LD₅₀ in a blood meal.

On the basis of serological studies it is suspected that WEE virus exists in Brazil in the Amazon valley [Causey & Theiler (26)] and in the lesser Antilles [Verlinde *et al.* (27)]. Hammon *et al.* (28) found no evidence of human infections in the Philippines. Although the virus has been repeatedly isolated from birds and mosquitoes in the southern United States, Schaeffer *et al.* (29) found no serologic evidence of human infection in a group of men likely to have been exposed. Libiková (30) has reported the isolation of WEE virus in Czechoslovakia, the first time it has been identified outside the western hemisphere.

Eastern equine encephalitis (EEE).—Evidence has accumulated during the last several years to incriminate the mosquito *Culiseta melanura* as one of the principal endemic vectors of EEE, isolants having been obtained from this species in Louisiana [Chamberlain *et al.* (31)], New Jersey [Holden *et al.* (32)], and Massachusetts [Feemster *et al.* (21)]. The isolations from all other arthropods number less than half of those reported from *C. melanura*. Karstad and his associates (33) reported isolations from *Aedes mitchellae*, *Anopheles crucians*, and a pool of *Culicoides* sp. Kissling (17) reported an additional isolation from *Anoph. crucians*, and Chamberlain (19) isolated EEE on two occasions from engorged *Culex salinarius*. Chamberlain *et al.* (34) estimated the infection thresholds for a number of mosquito species and classified them according to their transmitting efficiency. Species such as *Psorophora discolor*, *Aedes triseriatus*, and *A. aegypti* could become infected on blood meals containing 10^{3.0} LD₅₀, and could transmit with 50 to 90 per cent efficiency. Blood meals containing virus titers as high as 10^{8.0} LD₅₀ were necessary to infect even a small percentage of *Culex quinquefasciatus*, *C. salinarius*, and *Anoph. quadrimaculatus*, and these transmitted virus at less than 5 per cent efficiency. Chamberlain & Sudia (25) observed that *Culex tarsalis* was exceptional among *Culex* species in that it had an infection threshold of only 10^{2.5} LD₅₀ with EEE and could transmit this virus from bird to bird. Sudia *et al.* (35) showed that *Aedes sollicitans* could transmit EEE virus from horse to horse when the blood virus titer in the donor animal was slightly above the average observed in that species. Schaeffer & Arnold (36) suggested that "sylvan" and "urban" cycles of EEE virus maintenance may exist similar to those observed in yellow fever. Chamberlain (19) postulates that the "sylvan" cycle may be dependent upon *C. melanura* as a vector but, in view of the rarity with which this species feeds upon mammals, other species such as *Aedes sollicitans* and *Mansonia perturbans* are probably the vectors during epidemics involving horses and man. Isolations of EEE virus from naturally infected mosquitoes have been relatively few even during periods of demonstrated activity of this virus in birds [Wallis *et al.* (37); Kissling *et al.* (17)]. Chamberlain (38) estimated that infected *A. aegypti* usually inoculate less than 100 LD₅₀ although, in rare instances, an individual mosquito may inoculate as much as 10,000 LD₅₀. Chamberlain, Corrigan &

Sikes (23) have shown that 13 to 16 days at 80°F. are required for *A. aegypti* and *A. triseriatus* to reach the status of optimum transmission after feeding on virus titering $10^{7.5}$ to $10^{8.4}$ LD₅₀ per blood meal.

Kissling and his co-workers (39) demonstrated a high incidence of antibody to EEE in wild birds, and found that viremia levels in large birds such as ibis and egrets reached $10^{2.5}$ to $10^{3.3}$ LD₅₀, while smaller birds such as blackbirds, grackles, and English sparrows developed maximum viremia levels of $10^{4.5}$ to $10^{9.0}$ LD₅₀. Viremia in the larger birds lasted for 48 to 80 hr., while smaller birds had viremia periods up to 122 hr. Stamm (20) lists 24 isolations of EEE virus from wild birds in the eastern and Gulf states from 1950 through 1957, the earliest isolation being made in March and the latest in September. In a year-round sampling of an endemic area in Louisiana, Kissling *et al.* (17) isolated EEE virus from wild birds as early as March 19. Serological studies revealed a higher antibody rate in those bird species migrating to the tropics for the winter. However, these workers (40) failed to find support for the hypothesis of annual reintroduction of the virus into the United States by migrating birds, because no virus was obtained from over 1500 birds that were winter residents of southern Florida and spring migrants along the Louisiana coast. In fact, the principal migration waves appeared one to two months later than the demonstrated activity of EEE virus in birds in Louisiana. This fact plus the antibody studies suggest that fresh-water swamps serve as permanent foci for the EEE virus. Outbreaks of EEE in ring-necked pheasants reared in captivity in Connecticut have been described by Luginbuhl *et al.* (41), and show peculiar epidemiologic patterns in that contact transmission is suggested. Holden (42) demonstrated that pheasants could be infected by ingestion of the virus. Satriano *et al.* (43) were unable to infect pheasants by feeding virus contained in capsules, or by instilling virus into the palatal cleft, but were successful when it was introduced into the crop by tube. They found virus in the quills of subcutaneously and intratracheally inoculated pheasants to the sixth day after inoculation, a period slightly longer than the period of viremia. They also showed that EEE was spread among pens of pheasants exhibiting cannibalism, and that such transmission was prevented by debeaking the birds.

Kissling and his colleagues (44) found maximum viremia titers in horses experimentally infected by the subcutaneous route or by the bite of infected mosquitoes to be $10^{6.5}$ to $10^{4.6}$ LD₅₀, with viremia periods of 24 to 66 hr. Lethal infections in horses were not produced by inocula containing less than $10^{3.3}$ LD₅₀. These observations along with those of Sudia *et al.* (35) indicate that while horses may occasionally serve as a source of virus for infecting mosquitoes, the majority of infected horses are dead ends for EEE virus.

Epidemics of EEE in man are usually quite limited in respect to both overt and inapparent infections. Schaeffer *et al.* (29) found only 4 of 51 men with EEE antibody, even though they had worked as lumbermen for periods of several years in an endemic swamp. Feemster *et al.* (21) found only 2 of 356 persons living within one mile of a known endemic focus in Massachu-

setts to have antibody to this virus. Causey & Theiler (26) found 13 of 314 sera collected from human residents of the Amazon valley to have neutralizing antibody for EEE. Hammon, Schrack & Sather (28) found no EEE antibody in humans in the Philippine Islands, although approximately one-third of the horses sampled had such antibody.

Jungherr & Wallis (45) suggest that the extensive development of suburban woodland in New England, together with the encouragement of wild bird life, may enhance the problem of EEE.

Venezuelan equine encephalitis (VEE).—Sanmartin-Barberi (46) described an outbreak of mild febrile illness in humans in Espinal, Colombia, during March to June, 1952. Seventy cases occurred, none of which was fatal. Only one demonstrated signs of encephalitis. VEE virus was isolated from the blood of two patients.

Chamberlain and his associates (47) experimentally inoculated six species of birds native to the southeastern United States with VEE and found them to respond with viremia but without clinical signs. The viremia lasted generally for four days with maximum titers ranging from $10^{3.0}$ LD₅₀ to only traces in undiluted blood. Pigeons and mourning doves developed low blood virus levels. Transmission could be effected among birds with *Aedes triseriatus* as a vector. The infection threshold for *A. triseriatus* was about $10^{2.5}$ LD₅₀ of virus, and all tested members of this species became infected after ingesting blood meals containing virus titers of $10^{4.8}$ LD₅₀.

Kissling *et al.* (48) produced lethal infections in horses with as little as 32 LD₅₀ inoculated subcutaneously, and these animals were infected by intranasal instillation of the virus, by bite of infected mosquitoes, and by contact with infected horses. Virus was detected in the eye, mouth, nasal washings, milk, and urine of infected horses and the viremia was more prolonged and of higher titer than that observed in birds. Transmission from horse to horse was successful, using *A. triseriatus* as a vector. The disease in horses was not consistently encephalitic. It was proposed that mammals may be the natural hosts of this virus.

Causey & Theiler (26) observed antibody to VEE in 5 of 30 workers at the agricultural experiment station near Belem, Brazil.

Mayaro virus.—A virus infective for suckling but not for adult mice was first isolated from the blood of five humans suffering from a mild febrile illness in Trinidad [Anderson *et al.* (49)] during August and September, 1954. Four of these patients resided in a heavily forested area of Trinidad, while the fifth was a resident of Port of Spain. This virus was shown by Casals & Whitman (50) and Downs & Anderson (51) to be a member of the Group A arthropod-borne viruses, and was named Mayaro. Marked cross-reactions even by neutralization tests in mice, were observed between Mayaro and Semliki Forest virus. In April and May of 1955, Causey & Maroja (52) investigated an epidemic of febrile illness occurring on the River Guama in Brazil, and obtained six additional strains of Mayaro virus from the blood of patients. The first four isolants were from new residents of the area, which

was located on the edge of a relatively unexplored forested country. The disease occurred among forest and quarry workers. A serologic survey of the residents showed a 21.7 per cent incidence of antibody in males and a 9.7 per cent incidence in females. Antibody to Mayaro virus was also demonstrated elsewhere in the Amazon valley, and these authors suggest that the previously reported antibody to Semliki Forest virus found in residents of the Amazon basin may have been in reality antibody to Mayaro. A third outbreak of illness caused by Mayaro virus was reported by Schaeffer *et al.* (53) among Okinawan colonists in Bolivia. These cases likewise were observed in an area bordering upon virtually unexplored forest country. The agent was isolated from the blood of a febrile woman. They estimated that approximately 10 per cent of the 403 recent cases of febrile illness were due to infection with this virus. *Culex quinquefasciatus* was the most common mosquito of the area, although phlebotomus, flies, ticks, and mites were also present. No isolations have been reported from arthropods. Although no evidence of infection in other vertebrates is available, all three areas have monkeys in the habitat. Aitken & Anderson (54) obtained transmission of the virus by *Aedes scapularis* after parenteral inoculation into the mosquito, but failed with *A. serratus*, *C. quinquefasciatus*, or *Mansonia wilsoni*.

Sindbis virus.—A new virus belonging to Group A was first isolated in 1952 in Egypt by Taylor *et al.* (55) from a pool of *Culex pipiens* or *C. univittatus*, or both. Subsequently, they made 12 additional isolations from mosquitoes and one from a hooded crow. They determined the size of the virus by gradocol membrane filtration to be approximately 40 to 48 μ . The agent was lethal for suckling mice but did not consistently cause fatal infections in mice older than 10 days. It was pathogenic for chick embryos. Monkeys and several species of birds were shown to become viremic but not obviously ill after experimental inoculation. It was shown that the virus could be transmitted by both *C. pipiens* and *C. univittatus*. Antibody was found in 14 per cent of humans under 14 years of age, and in 34 per cent of those over 15 years, all residents of the Nile delta. Nine per cent of the wild birds and 52 per cent of the domestic quadrupeds had Sindbis neutralizing antibody. The virus has also been isolated in South Africa from culicine mosquitoes [Kokernot *et al.* (56)].

GROUP B VIRUSES

St. Louis encephalitis (SLE).—In the United States, where the only human cases of encephalitis due to the SLE virus have been reported, two epidemiologic patterns are observed [Chamberlain (19)]. In the western part of the country it is essentially a disease of rural inhabitants, while in the mid-western United States it is an urban disease. Longshore *et al.* (5) report that the first SLE infections of a season in California appear in July, approximately one month after the first cases of WEE are observed, and may be found somewhat later in the autumn than is WEE. There is no apparent age selection among the clinical cases observed in that state. Chin and associates

(57) described an urban outbreak in the lower Rio Grande Valley in 1954 in which there were 373 reported cases and an estimated total of 1000 cases. In this outbreak the higher age groups were attacked more frequently. The disease was observed at least three times more frequently in Anglo-Americans than in Latin-Americans. Following this epidemic, Sullivan, Irons & Sigel (58) reported that 70 per cent of a sample of sera taken from individuals who exhibited no illness contained antibodies to SLE. Razenhoffer *et al.* (59) described an increased incidence of both clinical and inapparent infections in the age group above 40 years during an urban outbreak in Kentucky.

The principal vector of the virus in California has long been known to be *Culex tarsalis*. Outside the range of *C. tarsalis*, mosquitoes of the *C. pipiens-quinquefasciatus* complex are also incriminated as vectors. These were the predominant species during the recent Texas and Kentucky outbreaks. The virus was isolated from engorged *C. pipiens* in Kentucky (59), and Beadle and his associates (60) reported two isolations from *C. quinquefasciatus* during the 1954 Texas epidemic. Anderson *et al.* (61) reported the isolation of the organism during 1955 in Trinidad from *C. coronator*, *C. caudelli*, and *Psorophora ferox*, these were the first isolations of SLE outside the continental United States. Galindo *et al.* (62) obtained three isolants from *Sabethes chloropterus* collected in Panama as well as another from a pool of *Sabethes* unidentified as to species. On two occasions they isolated the virus from the blood of men engaged in capturing mosquitoes. Chamberlain & Sudia (25) have shown that the infection threshold of SLE for *C. tarsalis* is near 10^0 LD₅₀. Chamberlain, Sudia & Gillett (63) compared the transmission efficiency of five geographically distinct strains of *C. quinquefasciatus* and three of *C. pipiens*. All transmitted a California strain of SLE with nearly 100 per cent efficiency. The incubation periods necessary for reaching optimum transmission efficiency were approximately 12 days for *C. pipiens* and 19 days for *C. quinquefasciatus* when both were held at 80°F. and 72 to 76 per cent relative humidity. All *C. pipiens* and *C. quinquefasciatus* strains showed about an equal degree of avidity for feeding on either man or chickens. In addition, these authors found that *C. restuans* and *C. salinarius* could transmit with 80 to 100 per cent efficiency, while *Aedes aegypti*, *A. dorsalis*, *A. triseriatus*, *A. vexans*, *Mansonia perturbans*, and *Psorophora confinnis* were also able to transmit but at greatly reduced efficiency. Sudia (64) compared the extrinsic incubation period of SLE virus in *C. pipiens* and *C. quinquefasciatus* after intrathoracic inoculation. Transmission efficiency reached 100 per cent in six days for *C. pipiens*, and in eight days for *C. quinquefasciatus*. Aitken (65), using a Trinidadian strain of SLE, was able to obtain transmission with *C. pipiens fatigans*, *Mansonia arribalzagae*, *M. venezuelensis*, and *Psorophora ferox*.

The St. Louis virus has been isolated from several species of naturally infected wild birds. Razenhoffer and his colleagues (59) reported the isolation of the microorganism from a flicker (*Colaptes auratus*) during the 1955 Kentucky epidemic. Downs *et al.* (66) isolated it from the blood of a rusty

dove (*Leptoptila verreauxi*) in Trinidad in 1956. Olitsky & Clarke (67) cite other isolations from birds, two in the United States and one in Haiti. Chamberlain *et al.* (68) succeeded in transmitting SLE to wild birds with *C. tarsalis*. Sudia & Chamberlain (69) compared the SLE viremia levels in chickens of various ages. Chicks 0.5 days old had maximum viremia titers of $10^{3.5}$ to $10^{5.0}$ LD₅₀, with viremia periods ranging to seven days. As the age of the chickens increased, the viremia periods became shorter and the maximum titers lower. Chamberlain *et al.* (68), using a California strain of virus, observed viremia periods of five to seven days in several species of wild birds. Kissling (15), using a Kentucky strain of virus in English sparrows, demonstrated longer periods of viremia, one of the birds being positive when tested on the third, eighth, sixteenth, and twenty-third day after inoculation. Birds show no clinical evidence of disease after infection with SLE virus, and Chamberlain (68) proposes that this benignancy of infection indicates a well-adjusted host-parasite relationship and suggests a long and close association.

Kissling (15) reviewed the unpublished experiments done by Stamm wherein he was able to demonstrate viremia in only one of four cottontail rabbits and in none of six roof rats inoculated with SLE.

The overwintering mechanisms of SLE virus are unknown. Reeves, Bellamy & Scrivani (7) have reported the only winter isolation, which was obtained in March from *C. tarsalis*. These workers (8) observed the persistence of the disease in experimentally infected *C. pipiens* for 31 days and in *C. quinquefasciatus* for 14 days when the mosquitoes were placed at 55°F. after allowing a 10- to 13-day preliminary incubation at 75° to 84°F. When infected *C. quinquefasciatus* were held in an unheated cellar during the winter (Kern County, California) the virus persisted in them for 116 days. Reeves *et al.* (10) reported isolating the organism four times in a four-year period from a total of 130,000 *Bdellonyssus sylviarum* and *Dermanyssus americanus* mites collected from bird nests in California. However, they were unsuccessful in initiating long-term infections in these mites under experimental conditions. Chamberlain *et al.* (70), in a quantitative study of SLE infections of *D. gallinae*, *Ornithonyssus bursa*, and *O. sylviarum*, could demonstrate detectable virus for less than two days in these mites after ingestion of a viremic blood meal. No transmissions could be obtained when these "infected" mite colonies subsequently fed upon normal chickens. There is no evidence that mites can serve as permanent reservoirs of SLE virus. Chamberlain & Sudia (25) could not demonstrate transovarian passage of SLE virus from infected *C. tarsalis*.

Causey & Theiler (26) have found SLE antibody in residents along the Amazon River. Hammon *et al.* (28) found the antibody in Filipinos only in the simultaneous presence of other Group B antibodies, and therefore were unable to say unequivocally that the virus exists in the Philippines. Although no clinical infections by SLE virus have been diagnosed in Trinidad, Anderson (71) suggests that this virus may be the cause of some undiagnosed

fevers on that island. Verlinde *et al.* (27) found neutralizing antibody in residents of Curacao who had recovered from encephalitis. Groot and his associates (72) found antibody rates as high as 30.9 per cent in Santander, Colombia.

Japanese B encephalitis (JBE).—Diseases contracted from nature are not necessarily limited to rural areas, as witnessed by the repeated epidemics of JBE in Tokyo, one of the most densely populated regions of the world (73). There is little doubt that the principal, if not the only, vector of this virus in Japan is *Culex tritaeniorhynchus*. The organism has been recovered repeatedly from this species, and infection of vertebrates is related directly to the time when maximum numbers of infected mosquitoes are present in a specific area, according to Buescher *et al.* (74). These authors also report that infected *C. tritaeniorhynchus* were found in largest numbers after the peak total mosquito population was declining, suggesting that infection of the mosquito occurs from an extra-mosquito source, *i.e.*, from viremic vertebrates. Such a pattern of infection would tend to rule out the occurrence of transovarian passage of the virus in mosquitoes, and this thought is supported by the failure of Buescher and his co-workers (74) to isolate JBE virus from eggs, larvae, or pupae collected in nature. Using *C. tritaeniorhynchus* as a vector, Gresser *et al.* (75) were successful in the experimental transmission of JBE from chicken to chicken, from pig to pig, as well as from pig to chickens, black-crowned night herons, and to two species of egrets. They found infection rates of 22 per cent in the mosquitoes following blood meals containing only $10^{0.4}$ LD₅₀ of virus. Fifty per cent infection rates were obtained after blood virus meals titering $10^{1.0}$ to $10^{1.6}$ LD₅₀, and to 94 per cent rates with titers of $10^{2.2}$ to $10^{3.3}$ LD₅₀. The virus in the transmitting mosquito generally titrated above $10^{5.0}$ LD₅₀, indicating multiplication within the mosquito tissues. The extrinsic incubation period varied from 9 to 34 days when the mosquitoes were held at 74° to 77°F. and at 80 to 90 per cent relative humidity. Depending upon the titer of virus in the blood meal, the transmission rate of *C. tritaeniorhynchus* varied from 17 to 92 per cent. Scherer *et al.* (76) compared the attraction of *C. tritaeniorhynchus* to various hosts. Traps on the ground baited with pigs attracted more mosquitoes than those baited with humans or black-crowned night herons. Likewise, the herons attracted more mosquitoes than did chickens, little egrets, tree sparrows, grey starlings, or dusky thrushes. *C. tritaeniorhynchus* could be recovered in traps placed 24 to 50 ft. above the ground, although no virus could be isolated from them. *C. pipiens* were more attracted to chickens than to wild birds. This species varied in zootropism from one area to another, while *C. tritaeniorhynchus* did not. Buescher *et al.* (74) reported two isolations of JBE virus from *C. pipiens*, but suggest that the virus was probably derived from recently ingested blood.

Hammon, Sather & McClure (77) tested the sera of 1222 wild Japanese birds, mainly from the island of Honshu, and comprising 44 species, for JBE neutralizing antibody. Antibody rates of 50 per cent or above were found in

house swallows, meadow buntings, little ringed plovers, common cormorants, dusky thrushes, and blue magpies. Black-crowned night herons showed an antibody rate of 43 per cent. Many species of passerines had antibody rates of 20 to 25 per cent, while no antibody was found in pigeons or several species of ducks. Positive sera were also found in birds from Kyushu and Hokaido. Buescher and colleagues (78), working in a five-year period, isolated JBE virus 35 times from black-crowned night herons, 16 times from plumed egrets, and 3 times from little egrets. Initial infections among these birds, whether determined by isolation of virus or by antibody studies, took place during the last half of July. The epizootic continued for a two- to four-week period. Since their studies were not designed to assay the importance of avian species other than these communal ardeids, it is difficult to say whether their observations reflect a truly enzootic condition or an explosive epizootic. Scherer, Buescher & McClure (79) suggested that the night feeding habits of the night herons left the nestlings unprotected during the hours when mosquitoes were most likely to be active, thus accounting for the higher infection rate in this species. They also observed blood-sucking nematode parasites in the esophagus and stomach of many of the herons and mention them as a possible vector for virus transmission. Gresser *et al.* (75) observed viremia titers of $10^{0.5}$ to $10^{1.8}$ LD₅₀ in experimentally infected egrets. Buescher *et al.* (80) inoculated chickens, black-crowned night herons, plumed and little egrets, and grey starlings with small infective doses. Viremia appeared on the second or third day and persisted for an average of four days. Young chickens developed a viremia more often than older chickens. The maximum titers they observed in chickens ranged from $10^{1.3}$ to $10^{3.3}$ LD₅₀.

Scherer and co-workers (81) found that 94 per cent of the young pigs in an endemic area near Tokyo developed antibody to JBE between late July and early September. In two instances, they were able to demonstrate viremia in trap-bait pigs. Due to the rapid replacement of the swine population, susceptibles detected among other domestic animals were considerably less than among pigs. Gresser *et al.* (75) and Scherer, Moyer & Izumi (82) found that viremia periods in experimentally infected pigs averaged four days, and maximum titers ranged from $10^{2.5}$ to $10^{3.3}$ LD₅₀. Scherer *et al.* (83) found antibody to JBE in only 2 of 503 wild rodents collected in an endemic area.

Whether mosquitoes may become infected from viremic humans is not known. Scherer (84) reported that five per cent of 254 Japanese children developed antibody to JBE during the late summer without showing clinical evidence of disease. They suggest that man's contribution to mosquito infection is negligible compared to that of pigs and birds.

La Motte (85) experimentally infected bats (*Eptesicus fuscus*) under simulated hibernation with JBE virus. While no viremia could be detected during hibernation, the organism remained latent for 107 days and appeared in the blood three days after the hibernating conditions were terminated.

He was able to effect transmission from bat to bat using *C. quinquefasciatus* as a vector. He was also able to induce *C. pipiens* to bite and to transmit virus to bats being held at 50°F. However, direct evidence is lacking to prove that mosquitoes feed on bats in nature.

Hammon and his colleagues (28) established the fact that 98 to 100 per cent of the inhabitants of two Negrito villages in the Philippines possessed neutralizing antibody to JBE. Filipino children in Manila aged 6 months to 14 years had JBE antibody rates of 3 to 17 per cent, while the rate was slightly higher in Filipino school children near Clark Air Force Base, 60 miles north of Manila. Verlinde (27) demonstrated JBE antibody in residents of Sumatra. Pond *et al.* (86) observed JBE antibody rates in humans of 74 per cent in Malaya and of 67 per cent in Borneo. High antibody rates were also seen in domestic animals in Malaya. Smithburn *et al.* (87) reported a low JBE antibody rate in residents of India but could not unequivocally say that the virus exists there since these individuals also had developed antibody to other Group B viruses.

West Nile fever (WN).—West Nile virus has been shown by Goldblum and his associates (88) and Marberg *et al.* (89) to cause a dengue-like disease in Israel, and by Taylor *et al.* (90) to be responsible for febrile illnesses of young children in Egypt.

Taylor (90) isolated the virus repeatedly from mosquitoes in Egypt. Nine of these isolations were made from *Culex univittatus*, five from *C. antennatus*, and three from *C. pipiens* and/or *C. univittatus* (probably the latter). No isolations were made from pools of mosquitoes containing only *C. pipiens*. These recoveries were made only during the months of July, August, and September. Although fleas, ticks, lice, flies, and mites were also tested, no virus could be found in them. In experimental transmission studies with *C. univittatus*, Hurlbut (91) determined that the infection threshold was about $10^{1.5}$ LD₅₀. When feeding upon blood meals titering $10^{3.5}$ LD₅₀ it was possible to obtain 100 per cent infection of the mosquitoes. A similar infection threshold was obtained for *C. antennatus*, and 100 per cent became infected following ingestion of blood meals containing $10^{2.5}$ LD₅₀. The species, *C. pipiens*, was slightly more refractory, as the 100 per cent infection level required blood meals of $10^{4.5}$ LD₅₀. Taylor and his associates (90) state that the extrinsic excubation period of *C. univittatus* could be as short as five days when the mosquitoes were held at 82.4° to 89.6°F.

In Israel, the mosquito *C. molestus* is suspected of being the principal vector of WN. Tahori *et al.* (92) found that the virus in the blood meal must exceed a titer of $10^{2.8}$ LD₅₀ for *C. molestus* to become infected, and that it regularly transmitted the virus only after ingesting viremic blood meals titering $10^{4.0}$ LD₅₀ or higher. The extrinsic incubation period of *C. molestus* was 7 to 28 days. Hurlbut (91) obtained a successful transmission using the tick *Ornithodoros savignyi*, but attempts made with *O. erraticus* and *Argas persicus* were unsuccessful. In a single test he found the mite *Dermanyssus*

gallinae to be refractory to infection. In addition, Taylor (90) was unsuccessful in obtaining transmission with lice (*Pediculus humanus corporis*), bedbugs (*Cimex lectularius*), fleas (*Xenopsylla cheopsis*), and flies (*Musca domestica vicina*). Hurlbut & Weitz (93) compared the feeding preferences of several mosquitoes of Egypt. *C. univittatus* was the most diverse in its feeding habits and was the most strongly attracted to birds of the species observed. *C. antennatus* fed most commonly on man and domestic animals. *C. pipiens* and *Aedes pharoensis* were the most anthropophilic. *C. pipiens* was dismissed as an epidemic vector since its population was reduced during July, August, and September, the months of peak incidence of WN infection. Taylor (90) observed that *C. univittatus* was rare in non-endemic areas.

Among vertebrates, WN virus has been isolated once from a hooded crow and twice from pigeons [Taylor *et al.* (90)]. Work, Hurlbut & Taylor (94) experimentally inoculated several common wild birds of Egypt with this agent. Hooded crows developed the highest levels of viremia, the maximum ranging from $10^{6.0}$ to $10^{8.0}$ LD₅₀. English sparrows had maximum viremia titers of $10^{3.0}$ to $10^{8.0}$ LD₅₀, while kestrels, buff-backed herons, and palm doves responded with lower blood concentrations. Crows and sparrows often died as a result of the infection. The occurrence of natural antibody among wild birds in Egypt was most frequent in English sparrows and crows; the rates were 42 and 65 per cent, respectively. Twelve to 28 per cent of individuals of other wild avian species had WN antibody, as did about 16 per cent of chickens. Taylor (90) has reported that experimentally infected young chicks, attained viremias titering $10^{4.0}$ to $10^{6.3}$ LD₅₀, but became refractory to infection after the third week of life.

Taylor (90) found high rates of occurrence of antibody in horses (86 per cent), camels (78 per cent), and water buffaloes (72 per cent); the rate was lower in cattle, sheep, goats, and bats. Forty-three rats (*Rattus rattus*) tested were negative for antibody. After experimental inoculation with the virus, a mule developed blood virus titering less than $10^{-1.0}$ on the third day, while two sheep and a young water buffalo failed to reveal virus at all, but did develop antibodies.

These authors (90) investigated the distribution of antibodies among Egyptians living in the endemic zone and found it to be 44 per cent in those below the age of 15 years. Beyond this age the rate of occurrence was 72 per cent. Somewhat lower antibody rates were observed in an endemic area of the Sudan. In a year-round study in the Sindbis district the blood of 2824 febrile children was sampled for the presence of virus. Isolations were made each month from June through September. In Israel, Goldblum, Sterk & Jasinska-Klingberg (95) were able to demonstrate WN virus in 4 of 306 healthy individuals. They estimate that two-thirds to three-fourths of the summer fevers in Israel are caused by infection with WN virus. During an epidemic of WN infection in that country, Marberg and his colleagues (89) were able to demonstrate viremia in 13 of 70 patients.

Taylor (90) suggests that the virus overwinters in Egypt by a continuous but retarded passage between birds and mosquitoes through the three or four coldest months.

Ilheus virus.—De Rodaniche (96) reported the isolation of Ilheus virus from mosquitoes collected in Honduras in September, 1954. The mosquitoes consisted of a pool of 50 of the genus *Psorophora*, mainly *P. ferox*, but also including *P. lutzii* and *P. varipes*. The agent was isolated by de Rodaniche & Galindo (97) from *Sabethes chloropterus* collected in Guatemala in 1956. This represented the first finding of the Ilheus virus in Guatemala, and the first recovery from this species of mosquito. Anderson, Aitken & Downs (98) obtained the virus in Trinidad on two occasions, once from a lot of 75 mosquitoes consisting of eight genera, and a second time from a lot of 143 mosquitoes consisting of 3 species of *Psorophora*—*ferox*, *albipes*, and *cingulata*.

Aitken (65) was unable to effect transmission of Ilheus virus with *Aedes serratus* or *P. ferox* after natural feeding. However, after intrathoracic infection, *A. aegypti*, *A. scapularis*, *A. serratus*, *Culex quinquefasciatus*, *P. albipes*, and *P. ferox* all became transmitters [Aitken & Anderson (54)].

Causey & Theiler (26) found Ilheus antibody in 7 of 150 children aged 4 to 14 years, and in 36.2 per cent of 359 persons 15 years or older who were residents of the Amazon valley. The rate was higher in males than in females, suggesting an occupational exposure to the virus among men. Hammon *et al.* (28) found a few individuals in the Philippines with apparently specific Ilheus antibody.

Murray Valley encephalitis (MVE).—French and his associates (99) furnished definite proof that the virus of MVE exists outside of Australia when they recovered it from the brain of a fatal case of encephalitis in New Guinea. On the basis of finding antibodies in the sera of domestic fowl and humans, they deduced that infection took place approximately 30 miles inland from Port Moresby, Papua.

Rozeboom & McLean (100) tested several species of mosquitoes found outside of the known endemic area of MVE and found that *Culex tarsalis*, *Aedes aegypti*, *A. polynesiensis*, and *A. pseudoscutellaris* were all capable of transmitting the organism when allowed an extrinsic incubation period of 16 days following blood meals with an average virus titer of $10^{7.3}$ LD₅₀.

Yellow fever (YF).—A conference on yellow fever held under the auspices of the Pan American Sanitary Bureau in 1954 reviewed the current status of YF in the Americas (101). Yellow fever, during recent years, has spread across Central America, and in doing so has presented ecological features not previously encountered in South America. Trapido, Galindo & Carpenter (102) describe the forest east of the Canal Zone as a tropical rain forest typical of those found in the known YF endemic zones of the Amazon and Orinoco River basins. Here are to be found the known South American vectors of yellow fever virus, *Hemagoggus spegazzinii falco* and *Aedes leucocelaenus clarki*. The forest to the west of the Canal Zone is intermediate in character between the tropical rain forest with its mosquito stratification

and favorable conditions for YF transmission and the deciduous tropical forest which, with its periodic dry seasons, has been suspected of being less favorable as an environment for the maintenance of the disease. *H. spegazzinii* and *A. leucocelaenus* occur sparsely west of the Canal Zone and into Costa Rica. When sylvan yellow fever appeared in Costa Rica in 1951, Galindo & Trapido (103) designated *H. spegazzinii* as the responsible vector on an epidemiological basis. They reported that this species, normally an inhabitant of the forest canopy, descended to the ground in cacao plantations. They also noted the presence of *Sabethes chloropterus*. When the virus invaded Nicaragua, these workers (104) found *Hemagoggus equinus* to be the vector on the Pacific side and *Hemagoggus iridicolor* on the Caribbean side, again on the basis of epidemiologic observations. In July and August of 1954, howling monkeys were found to be dying of yellow fever along the north coastal plain of Honduras in an ecological situation described by Trapido & Galindo (105) as unlike those associated with other epizootics in Central America. In this case, no known vectors of YF were found in the area. They suspected *H. equinus* of playing this role, and again noted the presence of *Sabethes chloropterus*. After a prolonged dry period in Honduras, described as one of the longest and most severe in the history of the region, lasting from October, 1954, to July, 1955, Boshell & Bevier (106) observed that YF virus had weathered the unfavorable conditions and had progressed into Guatemala. Reports of dead monkeys, many with livers pathologically positive for YF, were received beginning in January, 1956. They note that *Cebus* monkeys, which survive YF infection, have their northernmost limits in Honduras, while the *Ateles* (spider) and *Alouatta* (howler) monkeys, which experience heavy mortality from YF, continue into Guatemala. In 1956, virus was isolated by de Rodaniche & Galindo (107) in Guatemala from *Hemagoggus mesodentatus* (14 times), *H. equinus* (3 times), and from *S. chloropterus* (4 times). Trapido & Galindo (108), during a mosquito survey in Panama, noted that *S. chloropterus* was the most frequently caught species throughout the study, using human beings as bait. They postulated that regular biting day after day, regardless of weather conditions, could be an important factor in disease transmission. Galindo (109) established a colony of *S. chloropterus* and made the following salient observations: it is diurnal, non-autogenous, and deposits one to two eggs at a time, preferring, in the laboratory, bamboo internodes containing water and an access hole. That time of development from egg to adult averaged about 24 days. The life span of female *S. chloropterus* was relatively long, averaging about 40 days. One-half of the females survived five to six weeks after their first blood meal.

Galindo and associates (110) obtained experimental transmission of YF virus by *S. chloropterus*, *Hemagoggus mesodentatus mesodentatus*, *H. mesodentatus gorgasi*, and *H. equinus*. De Rodaniche *et al.* (111) reported that *S. chloropterus* had an exceptionally long extrinsic incubation period of 28 to 43 days, and required a high titer of virus in the blood meal to become infected.

Rosen (112) experimentally infected marmosets (*Marikina geoffroyi*)

and white-faced monkeys (*Cebus capucinus*) with YF. The marmosets became ill, but two of three survived. The *Cebus* showed no clinical signs of infection.

In 1954, Trinidad experienced an outbreak of YF. Downs and co-workers (113) presented serologic evidence which indicated that the virus had not been present in this locale since about 1930. They presumed that it was introduced via Venezuela. Anderson & Downs (114) isolated the virus from seven dead or dying howler monkeys, and recoveries were also made from humans and from *Hemagogus* mosquitoes. These mosquitoes were captured at ground level, where they have not been ordinarily found elsewhere.

Yellow fever virus was obtained from mosquitoes caught in Panama in the fall of 1956 by de Rodaniche *et al.* (115). Two isolations each were made from *H. lucifer* and *S. chloropterus*, and one each was obtained from *H. equinus*, *H. spegazzinii falco*, and *Anopheles neivai*. Antibody to YF was found by de Rodaniche (116) in 7 of 44 juvenile spider monkeys collected in 1956, as well as in a juvenile marmoset caught west of the Canal Zone in 1956.

Causey & Maroja (117) reported two recent outbreaks of YF in Brazil, one in the Oriboca forest and the other near Belém. They were able to obtain 21 isolants from human blood specimens collected during these outbreaks.

Dengue.—In the western hemisphere, the dengue viruses appear to be transmitted only by *Aedes aegypti*. In Brazil, where this species has been eradicated, Causey & Theiler (26) found dengue antibody only in individuals over the age of 19 years. In the areas which they surveyed along the Amazon valley, type 1 antibody was encountered more frequently than was type 2. In Panama, Rosen (118) found no antibody to the dengue viruses in individuals born after 1942. He obtained serologic evidence that the 1941–42 epidemic was caused by type 2 virus; this is the first known etiologic association of this type with an epidemic. He found no dengue antibody in a test of 105 Panamanian monkeys. Serologic surveys in Trinidad indicated that no dengue has occurred there since 1954 [Aitken & Anderson (54)]. The recent absence of this disease is assumed to be the result of an eradication program directed against *A. aegypti*.

Although dengue had been endemic in the Society Islands, no cases have been reported after World War II. Rosen (119) tested 82 serum specimens from residents of these islands and found no antibody in persons born since 1944. Most of the sera from older persons contained type 1 antibody but only 2 of 21 sera examined by neutralization tests had type 2 antibody. Both of these individuals were over 50 years of age.

Rosen (112) found the following species of New World primates susceptible to dengue 1 and 2 viruses: *Cebus capucinus*, *Ateles geoffroyi*, *Ateles fusciceps*, *Saimiri orstedii*, *Aotus trivirgatus*, and *Marikina geoffroyi*. *Alouatta palliata* was susceptible to type 1 virus, but the one monkey of this species inoculated with type 2 did not develop the infection. Type 1 viremia was

demonstrated in the *Alouatta*, *Cebus*, and *Ateles fusciceps*, and viremia following inoculation of type 2 was demonstrated in *Ateles geoffroyi*.

Russian tick-borne viruses.—Considerable confusion exists as to whether the viruses represented in the Russian tick-borne complex are distinct entities or simply strains of the same virus. The clinical disease in man varies from relatively benign to severe febrile illnesses which may or may not be further complicated by meningitis, encephalitis, or a hemorrhagic syndrome. The more serious complications are often preceded by a milder meningitic syndrome, followed by a short period of apparent well-being (120). The epidemiologic features vary slightly from one clinical entity to another, but one feature, basic to all is the implication of Ixodid ticks as either direct or indirect vectors or as reservoirs of the virus. Omsk hemorrhagic fever in man occurs where there are sheep, but apparently the causative agent does not affect these animals. Louping ill, on the other hand, is primarily a disease of sheep and rarely of man. The differences observed may be due to the habits of the vector rather than to specific properties of the virus or host. Gajdusek (121) reports that *Dermacentor pictus* is probably the important vector of Omsk hemorrhagic fever, while *Ixodes ricinus* is the vector for louping ill. The principal host for *D. pictus* is the mouse, *Microtus gregalis*. The vectors of Russian spring-summer encephalitis vary geographically, being *Ixodes persulcatus* in the east and *I. ricinus* in the west. Outbreaks of biphasic meningo-encephalitis have been described in Yugoslavia and Austria [Bedjanic *et al.* (122); Grinschl (123)]. In both countries the disease was associated with tick bites [Kmet *et al.* (124); Richling (125)]. The agents isolated from the patients were shown to be strains of Russian spring-summer encephalitis virus [Pond & Russ (126); Vesenjaj-Zmijanac *et al.* (127); Verlinde *et al.* (128)]. The virus was also isolated from *I. ricinus* in Yugoslavia by Likar & Kmet (129). Occasionally, the organism is contracted by means other than the bite of infected ticks. Levkovich & Pogodina (130) have described an outbreak of disease in Russia occasioned by vacationers drinking raw goats' milk in areas known to be constant foci of Russian spring-summer encephalitis. Only 3 of the 102 patients observed had been bitten by ticks and most of the cases occurred in family groups. The virus was isolated from human and goat blood (131). Kyasanur forest disease virus of India has been isolated most frequently from *Haemophysalis spinigera* (12 times), but also once each from *H. turturis* and *H. papuana*. Although attempts were made to isolate Kyasanur virus from other genera represented in the endemic area which included *Rhipicephalus*, *Dermacentor*, *Amblyoma*, *Boophilus*, and *Ixodes* [Trapido *et al.* (132)], they were unsuccessful.

Although small wild rodents are considered to be the primary vertebrate hosts of Russian spring-summer encephalitis, Federov (133) has isolated the virus on five occasions from the brain and once from the blood of wild birds. The role of birds or of ticks in disseminating the virus, is unknown. The Kyasanur virus was first isolated from a wild monkey, *Presbytis entellus*

[Trapido *et al.* (132)]. Work *et al.* (134) report additional isolations of the virus from monkeys, as well as the presence of antibodies in wild rodents of the endemic area.

Pattyn & Wyler (135), using the Austrian strain of virus, found viremia in rabbits lasting for four days with maximum titers of $10^{0.5}$ to $10^{2.5}$ LD₅₀. Chickens circulated virus for five to eight days. Sparrows had periods of viremia lasting three to seven days with maximum titers reaching $10^{2.5}$ LD₅₀. Mosquito transmission attempts were unsuccessful. Virus could be demonstrated in *Culex molestus* for 18 to 24 hr. after the ingestion of a viremic blood meal. Virus could similarly be demonstrated for one to five days in *Anopheles maculipennis* var. *atroparvus*.

UNGROUPED VIRUS

Colorado tick fever (CTF).—Burgdorfer & Eklund (136) report the isolation of CTF virus from Columbian ground squirrels (*Citellus columbianus columbianus*), chipmunks (*Eutamias amoenus*), pine squirrels (*Tamiasciurus hudsonicus richardsoni*), a deer mouse (*Peromyscus maniculatus*), and from golden mantled ground squirrels (*Citellus lateralis tescorum*). This latter species yielded 28 isolants from 61 individuals tested from June through September, and it was the preferred host for the tick *Dermacentor andersoni*. In areas populated by golden mantled ground squirrels, *D. andersoni* revealed a 16 per cent infection rate. Other rodents sharing the habitat of this ground squirrel had higher infection rates than those found in areas lacking this species of tick. *D. andersoni* were collected in March before the rodents had emerged from hibernation. Practically no activity of adult *D. andersoni* could be detected after the latter part of June. Nymphs were found in June, July, and August, while larvae were found during July and August. Virus was isolated from this species of tick in March, May, and June.

Rozeboom & Burgdorfer (137) infected nymphs of *D. andersoni* by allowing them to feed on blood containing virus titring $10^{1.5}$ to $10^{4.5}$ LD₅₀. The virus was maintained by the ticks to the adult stage. No loss of organisms was seen when the ticks were held for 10 months or longer. Immediately after ingestion by the ticks, the level of virus remained stationary, however, increased concentrations were observed as the ticks moulted to adults, to the extent of 1.5 to 3.7 logs over the amount ingested.

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SYNCHRONOUS DIVISION OF MICROORGANISMS^{1,2}

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INTRODUCTION

In the past five years elaborate methods have been devised for the induction of synchronous multiplication in mass cultures of bacteria, protozoa, algae, and cancer cells. Four valuable review articles on cell synchrony have recently been published, discussing certain topics such as experimental systems, methods, applications, and the possible cellular mechanisms causing synchronization (5, 9, 46, 114).

Since the literature has already become too voluminous to permit a comprehensive analysis of the subject in the limited space permitted here, only certain aspects of the problem will be emphasized: those inherent in the concept of cell synchrony which have been the main concern of the author ever since he and Dr. E. Zeuthen established synchronization of cell division in the ciliate protozoan, *Tetrahymena pyriformis* (87). The core of the problem resides in the effect of the synchronizing agent upon the normal metabolism in the cellular life cycle. It is hoped that the elucidation of these effects will yield information on the nature of those processes which prepare a cell for division. As a corollary to this problem, there is an additional question of ascertaining the specific effects of physical and chemical means, all of which produce the same effect—synchronization of division.

Since the results of numerous experiments have become available, it is the author's intent here, perhaps still somewhat prematurely, to compare the available data on synchrony with the established knowledge of normal cellular metabolism. In addition, the author will hazard a working hypothesis concerning some controlling steps in those preparatory processes

¹ The survey of the literature pertaining to this review was concluded in January¹ 1960.

² The following abbreviations will be used: ATP (adenosine triphosphate); DI (division index); DPNH (diphosphopyridine nucleotide, reduced); GSH (glutathione, reduced); NTP (nucleoside triphosphate); SH (sulphydryl); SI (synchronization index).

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leading to cell division, with the hope that newer information will soon relegate it to the past.

This article is divided into the following sections: Introduction—with a brief discussion of the concept of cell synchrony; Terminology—including growth of cells and cultures, steady-state versus unbalanced growth, synchronization index, and natural versus artificial synchrony; Systems and Methods—reference to papers which have not been cited in previous reviews; Comparative Biochemical Effects of Synchronizing Agents—an attempt to screen the literature with an eye to similar effects in different systems (it is acknowledged that graphs and tables could greatly assist in elucidating much of the data, but space limitations have unfortunately precluded this); Summary—the postulation of possible controlling sites in the preparation of a normal cell for division and the proposal of a working hypothesis.

Concept of cell synchrony.—Over the last several decades much information has accumulated concerning the growth patterns and metabolic pathways existing in microbial systems. Such physiological or biochemical data are often expressed as the average amount, or as the average activity, per cell. Although the single cell is a convenient unit for comparison, it should be realized that the cell on which the data is based represents an "average cell," a cross-section through the microbial population. This average cell, depending on the physiological state of the culture, covers a smaller or wider part of the physiological and biochemical spectrum through which an individual cell passes in its life cycle. This is the principal reason why relatively little is known about the biochemistry of the normal life cycle of the individual microbial cell.

There are consequently two possible approaches in studies of the life cycle of the individual cell. First, the methods for the study of the metabolism of single cells may be refined; this rewarding approach has been followed to great advantage in recent years with newly developed cytochemical techniques. Second, standard biochemical procedures are used on large numbers of cells which appear to be in the same metabolic state of their life cycle; that is to say, a culture is used in which all the cells are "synchronized" with respect to some visible metabolic phenomenon, with the tacit assumption that they are biochemically identical. This postulated identity is difficult to prove and our most satisfactory "yardstick" for such a demonstration in mass cultures is simultaneous cell division, the only phase in the life cycle of the individual cell which can readily be observed.

The idea of cell synchrony being a tool for the biochemical study of the "life history" of the individual microbial cell was the motivating force in the search for a possible means by which to establish synchronization, and "stimulated by the work of Scherbaum and Zeuthen (1954) and of Hotchkiss (1954) several attempts have been made during the last few years" in this direction (46). For several decades many workers have observed and described so-called fission waves, rhythmic reproduction, mitotic "flare-ups" and bursts of cell division in microbial cultures, and in tissues of animals and

plants; none of these systems, to the author's knowledge, has been utilized for biochemical study. However, embryological material, in this respect, has been studied [e.g. (70)]. For more detailed information the reader is referred to Zeuthen's review article (114). Even if some of these reproductive rhythms are not as striking as the induced synchrony developed in recent years, it still seems appropriate to speak of cell synchrony, using the term "divison synchrony," in its broadest possible meaning, as any deviation from normal exponential reproduction, resulting in stepwise multiplication.

TERMINOLOGY

GROWTH OF CELLS AND CULTURES

No problem arises in speaking of growth or division in a single cell. In a mass culture, however, growth is often expressed as an increase in population number. There is good reason for doing so, but it should be recognized that such an approach is based on certain assumptions: whereas, in exponential multiplication, the average cell size might be almost constant (and such terms as "growth in mass" or "growth in number" serve the same purpose), examples from the literature demonstrate that such an accurate balance between growth rate (in mass) and division (in number) in microbial cultures apparently is quite rare (23, 24, 75, 114). Terminological confusion also arises in speaking of mass cultures in which the normal association of growth and multiplication is experimentally broken. Unless terms like single step growth, rhythmic growth, phasing of culture, synchronized cultures, and so on, are defined, doubt may exist as to their intended meaning.

STEADY-STATE VERSUS UNBALANCED GROWTH; SINGLE CELL VERSUS AVERAGE CELL

Considering a single cell, microbial multiplication involves a duplication of the structures and the machinery of the cell between two subsequent divisions. These processes occur randomly in an exponentially growing mass culture. If such a culture is chemically analyzed at specific time intervals during exponential multiplication the cellular composition is usually found to be constant (61). This is an example of "balanced growth," or of a steady-state, in metabolism. Based on these observations, Northrop postulated that "if any substance in the cell increased at a faster rate than the cells, no matter how slight, sooner or later the cell must be destroyed; while if it increased at a slower rate, sooner or later the last molecule would be lost and a new kind of cell appears which contained none of the slow growing component (61)." The steady-state, with its constant ratio of any component of the cell is, accordingly, explained by the synchronized reaction rates producing these substances. But, such an explanation can only pertain to the average cell in the mass culture, and it should be stressed here that this concept can, in no way, imply synchronized reaction rates for different compounds within an individual cell. As has been pointed out above, the average cell represents a hypothetical cross section through the life history of the single cell and can-

not, therefore, be equated with the cell as an individual. Such individuals are distributed over the generation cycle in proportions which follow certain mathematical laws (49, 85, 106).

Growth studies with the thymine-requiring strain 15T of *Escherichia coli* have led Barner & Cohen to the useful concept of unbalanced growth, in which the normal growth pattern of DNA and RNA syntheses is disturbed. Growth, consequently, is said to be balanced over a time span if the chemical composition of the average cell remains constant during this time (2, 9) and "steady-state" and "balanced growth" both have the same connotation in discussing synchronous cell division.

SYNCHRONIZATION INDEX

Although we have defined synchronous division in the broadest possible sense, as "any deviation of cellular multiplication resulting in a stepwise increase in population number," nothing on the basis of this definition can be said about the degree of synchrony. The need for a quantitative expression of synchronization has previously been realized (9, 114) and attempts to express the per cent of synchronization or phasing have already been made (79, 80, 114). Obviously, two approaches are possible.

In analogy to the mitotic index, the division index (DI) can be calculated by dividing the number of cells in the visible stage of fission by the total number of cells counted in a sample. The DI, in fact, has been used to great advantage by many workers, but we must keep in mind that an increased DI does not necessarily indicate an accumulation of the cells into a phase, representing the same stage in their life cycle. The possibility also exists that fission may have taken up a greater proportion of the cell's life span than usual. This fact has actually been substantiated by growth studies in *Tetrahymena*, in which the DI and the growth rate (cell number) in mass cultures were compared (78). An extreme case of a high DI in the absence of a detectable synchronous multiplication was observed in the author's laboratory. By the application of sublethal temperature shocks, a "pseudosynchrony" could be induced in *Saccharomyces cerevisiae* (45); after budding of the yeast cells has been blocked by the treatment in the two-cell stage for several hours, the cells gradually entered into a logarithmic phase of growth without any detectable synchronous multiplication. The other extreme is synchronous ("stepwise") multiplication without an accompanying high mitotic index. For example, cultures of human cervical carcinoma cells (strain HeLa) have been exposed to a temperature of 4°C. for 1 hr. and reincubated at 37°C. Under these conditions no cell division occurred for 17 hr., at which time 95 per cent of the cells divided within 1 hr. In such an experiment the mitotic index never exceeded 8 per cent, compared, for example, to 3 per cent in controls. To describe this phenomenon, Newton & Wildy have introduced the useful term "parasynchronous division" (59).

These few examples show that if the degree of synchronization is esti-

mated by division indices alone, hazards such as those encountered in pseudo-synchrony or parasynchrony might occur.

A second method for expressing the degree of synchrony is based on the actual change in cell number in the population, as in an equation given by Zeuthen for the calculation of the per cent of phasing. Here, the time, during which synchronization of half of the population occurs, is considered (114). As Zeuthen points out, this formulation is not complete, because the fraction of the population undergoing synchronous division has to be given separately. The problem of comparing adequately the degree of synchrony in such diverse systems as bacteria, protozoa, and cancer cells still remained. Therefore an analysis of the factors involved in formulating a quantitative expression of the degree of synchrony has been carried out and an equation derived, based on simple geometric considerations. From this equation a synchronization index (SI) has been computed for the estimation of the degree of synchrony in various microbial systems (79, 80).

NATURAL VERSUS ARTIFICIAL SYNCHRONY

Originally, successful synchronization of division in *Tetrahymena* was achieved on the assumption that a block applied to the cellular growth processes at a definite point in the cell cycle should cause the cells to "accumulate" at this particular point (87). However, studies of the temperature effect on multiplication in mass cultures and in single cell experiments (76, 103) confirm earlier observations that cell division, rather than growth, can be more readily suppressed by environmental changes (95). These findings support the hypothesis that growth and cell division are separate, but interdependent processes. Further substantiation has been provided (114). Changes in environmental conditions that induce synchrony such as temperature, light, and food can apparently upset the normal sequence of events occurring within the cell. Consequently, the metabolic changes that take place in synchronized cells are very likely not a biochemical magnification of the metabolic events in a normal single cell. It seems proper then, to question how abnormal, artificial, or pathological is such a system, and to consider, in these circumstances, the advantage of using a system of synchronized cells in order to understand the normal life cycle? The exponential multiplication of a microbial culture in a chemostat, operating under well-controlled conditions, may be considered as normal. Such a definition of normality is a legitimate matter of convenience, but ecological factors, such as diurnal and seasonal changes in temperature, light, and food within the environment, should not be neglected (4, 18, 21, 30, 65). The cells must possess certain regulatory mechanisms that are able to cope with changes of this nature. All of the many naturally synchronous systems described in the literature are an expression of biological systems responding to such environmental changes as those mentioned. Are such regulatory responses of the cell to their environment pathological? The unique advantage of experimentally

synchronized cells lies in the possibility of obtaining sufficient material for studying cellular control mechanisms that might exert their full effects even in the presence of only catalytic amounts of their metabolites. Prusoff's suggestion, as to the role of a thymidine-containing cofactor in the control of cell division, illustrates this point (68). Inhibitors have also proven to be a valuable tool to the biochemist in elucidating the reactions of the intermediary metabolism. By analogy, it is felt that the inhibition of certain processes inducing synchrony will ultimately shed more light on the metabolism of the normal life cycle of an individual cell.

SYSTEMS AND METHODS

For the experimentalist working in the field of cell synchrony, a critical evaluation of the systems studied and the methods used to date would be of value; however, such an attempt is beyond the scope of this paper. Only those synchronized systems developed recently, but not mentioned in previous reviews, will be cited here.

Campbell (8, 9) discusses 18 microbial systems that have been synchronized by appropriate "single shifts" in illumination, growth temperature, or composition of growth medium. A brief change in an environmental factor from the optimum and a return to the optimum (that is, a "double shift" or a "single shock") produces similar results. Characteristic of such single or double shifts is the induced "tuning" of cell division to the environment, but such shifts often induce only one synchronous division. By repeating the shift or shock within the subsequent generation, synchronous division can be extended over several generations ("periodic treatment"). If the shocks are so spaced in time that the cells have no chance to enter into division and continue to grow, it is sometimes possible to produce additional synchronous division cycles without further treatment ("prior treatment") (87, 88).

A periodic temperature treatment which induces synchrony of cell division in *Amoeba proteus* has recently been standardized by James on the basis of earlier observations (30). Diurnal temperature shifts were found to produce synchronous division in the flagellate, *Astasia longa* (63); and temperature-induced synchrony in cultures of the trophozoites of *Endamoeba terapinae* (71) and *Corynebacterium diphtheriae* (72) have also been reported.

Appropriate nutritional shifts during growth of *Saccharomyces cerevisiae* were used for synchronization of multiplication in the study of peptidase and proteinase activity (98, 99, 100, 102). Barner & Cohen's method of "differential starvation" (2) for the induction of synchrony in an *Escherichia coli* mutant has been applied to *Lactobacillus acidophilus* R-26 (7), which has a specific requirement for deoxyriboside or deoxyribotide. The addition of thymidine to a thymidine-starved culture also results in division synchrony (7).

An additional method for the induction of synchrony in *E. coli* has been developed in which a combination of temperature shifts and food shifts is used (91, 92). This system was employed for the study of RNA synthesis in

the presence and absence of 4-mercapto-pyrimidine (90) and for the respiration rate during synchrony of division (64). Yanagita observed synchrony in *Schizosaccharomyces pombe* upon transfer of a stationary phase culture into fresh medium (110, 111). X-irradiation of exponentially growing cultures of *Saccharomyces cerevisiae* induces synchronous budding. This system was used for studies of the uptake of P^{32} into various fractions (93).

Reports on successful light-induced synchrony appeared after the accidental observation of division bursts during studies of efficiency of photosynthesis in *Chlorella* (101) and during the analysis of light flashes produced by *Gonyaulax polyedra* (22, 97). Cycles of illumination were also used to obtain synchrony of division in *Euglena gracilis* (11), *Chlamydomonas moewusii* (3), and *Chlorella pyrenoidosa* (42, 43, 89). The prior treatments of the type developed for *Tetrahymena* by Scherbaum & Zeuthen (87) have been applied to studies on lipid content in relation to cell division (15); changes in free amino acid and protein concentration during cell growth and synchronous division (10, 56, 81, 94); nucleic acid synthesis (29, 54, 55, 74, 77, 84); effects of chloramphenicol and azaserine on cell growth and cell division (40); changes of SH-containing compounds in relation to cell division (81, 83, 94); and phosphates (66, 67, 72, 84, 86) and carbohydrate content (31) in synchronized cells.

Holz *et al.* have established a similar temperature pretreatment for *Tetrahymena pyriformis*, mating type I, variety 1 (26). Harrington successfully extended this method by growing this strain in synthetic medium and has followed DNA metabolism during cell division, comparing untreated and ultraviolet-irradiated specimens (20). The reader is referred to the original papers for more details. The author has recently learned that mathematical aspects of division synchrony are also under study (113).

COMPARATIVE BIOCHEMICAL EFFECTS OF SYNCHRONIZING AGENTS

Although the literature contains many examples of the effects of synchrony-inducing agents upon both cellular structure and biochemical pathways, the mode(s) of action(s), despite much investigation, has not yet been determined. Aside from the question of identifying the mechanism(s) involved, the author considers equally important the problem whether or not all these agents act in the same way and on the same sites. In the following paragraphs, therefore, we will compare the effects of various environmental agents upon the amount and composition of known cellular constituents, such as proteins, amino acids, nucleic acids, and phosphates. On the basis of such a comparison, a hypothesis concerning probable sites of metabolic control as related to cell division, will be advanced (see summary).

Shifts in food.—Schaechter, Maaløe & Kjeldgaard (73) studied *Salmonella typhimurium* during balanced growth in a variety of media and found that cell mass (optical density), and DNA and RNA of the average cell are exponential functions of the growth rates produced by various media at a given

temperature (37°C.). For example, comparing growth rates and cell size in a glucose-salt solution and in meat extract-peptone nutrient broth, one finds a generation time of 52 min. in the former medium and 29 min. in the latter. At the same time, the cell size in the glucose-salt solution is one-half that found in nutrient broth.

When the cultures were transferred from glucose to broth, the total mass and rate of RNA synthesis increased immediately, while DNA synthesis and cell division continued at the previous rates for 20 and 70 min., respectively, before they changed to the rate characteristic for broth. This resulted in an increase in cell size. On the other hand, a transfer of the culture from nutrient broth to glucose-salt is characterized by a period of continued cell multiplication and DNA synthesis, for 20 and 10 min., respectively, before a change to the rate characteristic for glucose-salt occurs. No synthesis of either RNA or total mass occurred for 60 min. following transfer. When RNA and mass began to increase, the cells had already attained the size and composition characteristic for balanced growth in the glucose-salt medium. An orderly dissociation of the main synthetic activities apparently occurs, and this has been interpreted by the authors as evidence for the existence of separate rate-controlling mechanisms for growth and cell division (37). Under the conditions described, no synchrony of division occurred that might be attributed to the salt medium being too rich and not effectively blocking certain pathways in the preparation of the cell for division. Related experiments using protozoa have been reported by Kimball *et al.* and will be discussed later (36).

More drastic nutritional shocks (double shift) seem to be quite effective in producing synchrony. It was found that cell division and DNA synthesis are blocked immediately in the thymine-requiring mutant *E. coli* 15_T upon transfer to a thymine-free medium. RNA and protein synthesis were apparently unaffected (2). After an optimum of 30 min. of starvation, thymine was added and after a lag of 35 to 40 min., nearly all the cells divided synchronously.

Other workers using nutritional shifts in conjunction with a single temperature shock, were able to induce synchrony in *E. coli* B (91). Cells from the exponential growth phase were exposed to a cold shock (45 min. at 6°C.) and transferred to fresh medium. Seventy minutes after inoculation, all cells divided within a 10-min. period—the normal generation time being 45 min. If resting cells from the early maximum stationary phase of growth (when glucose has been depleted) are subjected to the same treatment, the length of the lag phase preceding synchrony is almost doubled. During this prolongation, the RNA content increases 100 to 500 per cent, while the DNA content increases only 20 to 100 per cent. The stepwise increase in cell counts was not reflected in the turbidity, which increased in an exponential fashion immediately after inoculation (91). These experiments with *E. coli* furnish results similar to those in the experiments on *Salmonella*, and indicate that DNA and cell division are more inhibited by the treatment than are cellular mass and RNA synthesis.

Shift in temperature.—The temperature of a culture of *Salmonella typhimurium*, previously grown for ten generations at 25°C., was raised to 37°C. (6). Cell mass (turbidity) rises immediately with the new rate characteristic for normal growth at 37°C. The viable cell count, however, seems unaffected by the temperature shift during the first 20 min.; but after 30 min. following the temperature shift, a slight synchrony is observed. A temperature shift evidently exerts differential effects on multiplication and on growth in mass cultures. Similar differential effects on particular synthetic activities were also observed by Falcone & Szybalski, who transferred cultures of *Bacillus megaterium* from optimal to low temperatures (17). With a single temperature change from 34° to 15°C., cell division was completely blocked within 30 min. for 4 hr. Turbidity and dry weight of the culture seemed to be least affected.

These examples indicate that the characteristic composition of the average microbial cell in a given steady-state is altered by shifts in nutrients or temperature, or both. Although a wide spectrum of responses is observed, depending on the organism studied and the degree and type of change, RNA and mass synthesis show a similar "tied together" response to such single environmental shifts, as do DNA and cell division. A transfer of a culture from suboptimal to optimal conditions (temperature, nutrients, etc.) results in an immediate increase in the rate of synthesis of RNA and mass, leaving DNA synthesis and cell division unaffected. And, conversely, a nutritional shift from optimal to suboptimal conditions gives different results: a change from a rich to a poor medium blocks RNA synthesis and the increase in mass, but not DNA synthesis and cell division. However, a temperature change from optimal to suboptimal conditions immediately blocks cell division, but not RNA and protein syntheses.

Single temperature shock (double shift).—From the above mentioned single shift experiments it is obvious that changes in growth medium or a shift in temperature, or both, is followed by adaptive changes of varying degrees (imbalance of growth) and finally by the re-establishment of balanced growth in the new environmental conditions. However, with respect to a double shift (single temperature shock), different results are observed.

A brief exposure of the culture to a shock like starvation, or high or low temperature, causes a temporary imbalance of growth followed by a "back regulation" to the rates prevalent under prestress conditions. In proper environments (temperature and time of exposure), synchrony of division can be induced by a single temperature shock, as illustrated in the following examples.

A 15-min. exposure of a pneumococcus culture to 25°C. followed by reincubation at 37°C., results in synchronous multiplication (27); while exponentially growing *Bacillus megaterium*, chilled to 15°C. for 30 min., divide synchronously upon reincubation at 37°C. after a brief lag (12, 17).

Similar growth characteristics were observed in *Salmonella typhimurium* grown at 37°C., when cultures were exposed to a single temperature shock of 25°C. for 30 min., and subsequently reincubated at 37°C. (39). A brief lag of

2.5 min. is followed by a period of synchronous multiplication during which approximately 50 per cent of the cells divide. The effects of a single temperature shock on the growth of *Tetrahymena* were studied by Zeuthen and his co-workers (103, 115), in which mass cultures grown exponentially at 22°C. were exposed to single cold shocks, ranging from 2 hr. to 16.5 hr. in duration. During exposure to the low temperature, there was a gradual decrease in the number of fission stages, indicating a temperature block of cellular and nuclear fission. Upon return to 22°C., a characteristic multiplication lag of approximately 2 hr. occurred, followed by a burst of division during which the population increased by 25 to 30 per cent. If such a culture is exposed to a second shock immediately after the peak of division, a second division synchrony results, similar to the "periodic treatment" of Maaløe & Lark (39, 47). It was once felt that such a periodic treatment producing environmentally "tuned" synchrony might increase the amplitude of the following division bursts (87, 115), but subsequent experiments with low temperature shocks did not verify this assumption; never could more than 35 per cent of the cells be observed in simultaneous fission. A similar approach with high temperature shocks was recently made (104). When cells grown at 27°C. were exposed to single temperature shocks of 33°C. for half an hour, each shock well timed to occur right after each new division maximum, the culture could be kept in division synchrony as long as ample nutrient supply was available. Such experiments suggest the usefulness of the chemostat in the study of synchrony. The degree of synchrony resulting from the application of one heat shock per generation is quite similar to the results obtained with cold shocks: the degree of division synchrony in a mass culture of *Tetrahymena*, "tuned" with the temperature change of the environment, does not improve in time.

In contrast, observations on bacterial systems suggest a somewhat different situation. In cultures of *Salmonella typhimurium*, exposed to repeated cold shocks of 25°C. for 30 min., the degree of synchrony improves with time (39).

Repeated shocks ("prior treatment").—If the time interval between two successive shocks of the periodic treatment is reduced, the cells have no chance to enter into division, but nevertheless increase in mass under suitable conditions. This seems to be a clear-cut demonstration of a separation of the two basic processes—the preparation of the cell for division and the bulk increase in mass. An impressive example is found in *Tetrahymena*. Mass cultures grown exponentially at 29°C. are subjected to seven temperature shocks. Each shock, 30 min. at 34°C., is followed by a period of half an hour at 29°C. During this treatment the average cell mass increases two- to threefold as the result of continued growth without cell division. After termination of the treatment, a "recovery" period intervenes before the cells enter into two to three successive synchronous division bursts of decreasing intensities, followed by a return to the normal asynchrony of random multiplication (87). While the treatment exerts its full effect on cell division soon after the ap-

plication of the first shock, the rate of synthesis of the culture's total mass is the same as in the untreated control for 4 hr. and is, then, followed by a progressive inhibition of synthesis in the latter part of the treatment. At the end of the treatment cell multiplication is reduced to 2.5 per cent and total mass to 65 per cent of that which we would have found in a normal, untreated culture.

By such treatments the randomly dividing cells of an exponentially growing culture are aligned to a very high degree with respect to cell division. But synchronous cell division need not necessarily reflect synchronous cell growth. This possibility must be considered when experimental results on synchronously dividing cells are used in any attempt to interpret the growth of a normal single cell.

Ciliates, such as *Tetrahymena*, offer unique advantages for the study of mass change and the analysis of synthesis of the elaborate infraciliature, a "biological marker," during the treatment and the correspondingly induced division cycles. In randomly dividing mass populations of *Tetrahymena*, a characteristic distribution of cell size was found, representing all stages in the growth and division cycle of the individual cell (85). Furthermore, it could be shown that characteristic size distributions correlate with different phases of normal population growth. The early working hypothesis of Scherbaum & Zeuthen that the heat treatment inducing synchrony "accumulates the randomly growing cells at a specific stage of their cellular life cycles" characterized by a certain size was put to test to determine the location of this block. Originally, it was expected that the cells would become more uniform in size (75), but the experimental results clearly indicate that this is not the case; unexpectedly, the cells become less uniform in size. The larger cells, normally found prior to division, grow more on a percentage basis than do the small daughter cells. This finding has been interpreted in the light of Swann's "reservoir hypothesis" (95), as an interference of the temperature treatment in the formation and storage of high-energy compounds in preparation for cellular fission. This presumptive store of high-energy compounds should be depleted by utilization of its contents in pathways much less inhibited than those preparing the cells for division, as in the synthesis of cellular mass (75, 76). We will elaborate on this point in the discussion of the possible mechanisms inducing synchrony.

The fact that mass synthesis is enhanced more in the larger, than in the smaller, cells during the treatment, suggests the need for analysis of structural development of the individual cells. Such studies might yield information as to whether the larger cells have more cytoplasmic structures, such as the number of ciliary meridians which is normally constant and genus-specific, or the number of kinetosomes on the meridians; or whether this mass increase occurs on lower levels of organization not readily traceable by cytological studies. Williams & Scherbaum (109) showed that during treatment the number of cells with accumulated kinetosomes in the anarchic field for the second cytostome increases gradually, reaching 100 per cent just prior to

the end of the treatment. The number of kinetosomes on the ciliary meridians also increased. Kinetosomal counts were made on the comparable meridian at various stages in the treatment, and a strict duplication was seen to occur. The number of ciliary meridians was always constant and characteristic for normal cells. It was concluded that all cells, irrespective of their size, are aligned in these observed cytological respects and resemble a normal cell prior to fission, when the kinetosomes of the presumptive cytostome are not yet arranged into four membranelles of a functional mouth. Aside from the increased spacing between the ciliary meridians and the increased length of the cells, all cells seem to be normal and blocked at a specific point in their life cycle. Since maturation of the cells along this line of development appears to be normal, the "abnormal" increase in size of the larger cells must be due to an abnormal increase of other cellular structures or components on a different level of organization.

A specific block of the cytokinetic events was also demonstrated in a micronucleate strain of *Tetrahymena*, mating type I, variety 1, which was exposed to a series of five heat shocks, 43°C. for 30 min. each. It is noteworthy to mention that the optimum temperature for multiplication of this strain (32°C.) is close to the shock level for *T. pyriformis* GL (34°C.). Cytological examination of the cells at the end of the treatment show all micronuclei blocked in an anaphase-like configuration (26).

Single temperature shocks in single cells.—Although size distribution studies in *Tetrahymena* revealed that the older cells grow more than the younger cells during the exposure to multiple-shock treatment (75), no information was obtained on sensitive phases in the life cycle of the individual cell. Early observations of synchrony in mass cultures led to the assumption that the cells pass into a "most sensitive" phase prior to division (88). Conclusive experimental evidence was produced by Thormar in Zeuthen's laboratory (103). Single cells were isolated by capillary braking pipettes and the exact time of fission under well-standardized conditions was noted, thus establishing the exact time of birth of the daughter cells. At various known intervals after the completion of cytoplasmic fission, single cells were exposed to a temperature shock of 34°C. for 15 min. and returned to 28.5°C. Even if the period of heat exposure is subtracted in all cases, the subsequent cell division is delayed. This delay (50 to 55 min.) is relatively constant in the early part of the cellular life cycle and increases as the cell approaches the time for division. The results of these important findings were integrated with observations on synchrony by Scherbaum (76) and more recently by Zeuthen (114), and utilized for an explanation of cell synchrony in mass cultures. We will return to these interpretations in the concluding section.

PROTEIN AND FREE AMINO ACIDS

Free amino acids were analyzed in *Tetrahymena* in Agrell's laboratory by Christensson (10); and by the author and his co-workers (81). Scherbaum *et al.* were interested in only four characteristic growth stages: normal

exponential multiplication; the period after application of seven heat shocks when the cells are two to three times their normal size; the period just prior to synchronous fission; and in the early maximum stationary phase when multiplication has ceased. The over-all, normal picture of free and protein-amino acids was found to be retained during the treatment prior to division, and even during the maximum stationary phase no depletion of the free amino acids could be detected. The total amounts, during the treatment, increased by a factor of two to three, reflecting growth on the cytological level. Changes in unidentified compounds, perhaps SH-containing peptides, were noted at the growth stages mentioned, but they were not considered as being responsible for the induced fission block. This two- to threefold increase in free amino acids and the total protein content during the treatment was also observed by Christensson (10).

Christensson found that protein synthesis was blocked during the temperature shocks, but that it occurred at the optimum temperature between successive shocks. Therefore, it was concluded that synthetic activity proceeds in distinct waves, corresponding to the imposed temperature cycles. The protein metabolism during the heat treatment could be divided into three phases. The first one covered the temperature treatment until the seventh shock. This was characterized by a rapid synthesis of structural proteins between successive temperature shocks. The events occurring during the last (eighth) heat shock were considered as the second phase. While a complete block of the synthetic activity was observed during the preceding seven shocks, protein synthesis apparently occurs during this last (eighth) shock. Christensson suggests that this may be the result of an increased protein stabilization, or an adaptation commonly found in biological materials. During the third phase, the recovery period between the end of the last heat shock and the first synchronous division, the soluble proteins increased about 40 per cent, probably at the expense of the insoluble proteins, since they showed a simultaneous decrease. Christensson's interpretation, that adaptive protein stabilization might occur during the last heat shock, agrees with earlier observations: if a culture of *Tetrahymena* is exposed to 10 to 15 temperature cycles some cells gradually do enter the visible stage of cellular fission during the temperature treatment (80). Christensson's findings, that the amount of total protein per average cell remains constant during the first 4 hr. of the temperature treatment, seem to be incompatible with other observations of increases in cell volume, reduced weight, and dry weight occurring during this period (76, 84, 87).

Incorporation of C^{14} from labeled alanine and S^{35} from labeled methionine into proteins of synchronously dividing *Tetrahymena pyriformis* W has been reported (56): samples of the experimental cultures were incubated with the radioactive amino acids for 15 min. at various stages during and after the intermittent heat treatment. The specific activity of the protein fraction was reduced during the 7.5 hr. of temperature cycling to about $\frac{1}{4}$ th of that of the control. However, an increase of activity was observed

during the subsequent return of the culture to normal conditions. These results are in keeping with the findings of Christensson (10) and of Scherbaum *et al.* (81). Obviously, more detailed studies along these lines would be rewarding.

Using the "fast green" method, the histone content in normal and synchronized *Tetrahymena* was followed cytologically (84). While only the nuclei of cells in the maximum stationary phase appeared stained, the cells of the exponential phase of growth did not respond as uniformly; only 5 per cent of the cells showed stain in the nucleus alone, but in the remaining 95 per cent of the cells, dye was found in both the nucleus and in the cytoplasm. Following the heat treatment the cells were deeply stained both in the nucleus and cytoplasm. The results of these observations are not yet understood, since the nature of the dye-binding sites in the cytoplasm is not known.

NUCLEIC ACIDS

In most of the many systems which have been synchronized thus far, nucleic acid metabolism was studied with the hope of learning more about the connection between RNA and protein synthesis, and the relationship of DNA and cell division. As it is not the intention of the author to review all of these studies, attention shall be focused on only a few significant examples.

Effect of a single shift on nucleic acid composition.—Several metabolic studies on nucleic acids in microbial mass cultures under changing environmental conditions, have recently been reported, such as nutritional shifts in *Salmonella* (37), starvation in *E. coli* 15T (91) and *Bacillus megaterium* (28). These experiments suggest a dissociation of synthetic activities causing an imbalance of growth in the average cell of a mass culture. Support for this conclusion exists in cytological and biochemical findings. For example *Bacillus megaterium* was grown at 34°C. and exposed to a single temperature shock of 12°C. for 30 min.; after a brief lag, the culture underwent synchronous multiplication upon reincubation at 34°C. (28). This cold shock produces striking changes in the appearance of the nuclei. Their size and optical density, when compared with the untreated controls, increases considerably. Observations such as this suggest that the cells accumulate in the same abnormal state. In *Tetrahymena*, exposed to eight sublethal temperature shocks, similar increases in nuclear size have also been observed (115).

It should be recalled that in *S. typhimurium*, the nutritional shift from glucose-salt medium to a peptone broth caused a characteristic dissociation of DNA and RNA synthesis (37). In the average cell, RNA is synthesized immediately at a new rate, while DNA increases with the pre-shift rate for as long as 70 min., when it is stepped up to the new rate. *Salmonella typhimurium* grown in broth at 25°C. and transferred to 37°C. shows a similar pattern with respect to changes in RNA synthesis, but differs in respect to DNA synthesis. In contrast, DNA synthesis seems to be stimulated by the temperature shift, increasing approximately 75 per cent within 8 min. (normal

generation time being 20 min.) (39). This phenomenon has been explained by assuming that DNA synthesis in the pre-shift cells had been phased by the temperature change and that the rate of DNA synthesis in individual cells was not increased over the normal value. The recent observations that DNA synthesis might be continuous throughout the normal division cycle (1, 112), offers an alternative explanation which is favored by Maaløe (46). Assuming a continuous synthesis of DNA between subsequent divisions, the increase in DNA synthesis in the culture following the temperature shift can only result from an increase in the rate of synthesis in each cell. Maaløe assumes that the temperature shift disrupts the normal control which determines the rate of DNA synthesis and, for a short time, allows this process to proceed uninhibited.

Effect of single shocks.—The similar pattern of responses to either a nutritional or temperature shift in *S. typhimurium* resulting in an immediately increased RNA synthesis rate, leads to the question of whether a simultaneous shift in nutritional and temperature changes shows a synergistic effect. From work on *E. coli* B, some information has become available (90). The cells were grown in a synthetic medium containing glucose, washed and re-suspended in a glucose-free medium, and exposed to a single temperature shock of 5°C. for 45 min., and then inoculated into synthetic medium at 37°C. Three minutes after the inoculation, glucose was added. During the cold shock, RNA synthesis was observed to be blocked but upon transfer to a temperature of 37°C., rapid RNA synthesis took place, seemingly unenhanced by the addition of glucose. However, an interesting change in an acid-soluble RNA extract was found during cold exposure. This fraction was reduced to less than half during the cold shock, increased within a few minutes to the original value following inoculation at 37°C., and seemed to be elevated further by the addition of glucose. Within 45 min. after the temperature change, it had increased tenfold. (This final value was four times the amount found in the normal control.) Approximately 70 min. after the temperature change, synchrony of division occurred. In addition, changes in RNA fractions have been correlated with growth patterns observed in *E. coli* (105).

Nucleic acid synthesis in multishock systems.—It seems logical to assume that the disturbed pattern of macromolecular synthesis produced by single nutritional or temperature shifts, or both together, might be even more pronounced after an intermittent multishock treatment. Evidence from several laboratories shows that this is probably the pattern for nucleic acid synthesis of *T. pyriformis* W (20, 29, 54, 55), and for *T. pyriformis* GL (77, 84, 115). These results are not unequivocal, presumably due to variations in isolation procedures and to strain differences. The base ratios in *T. pyriformis* GL of RNA and DNA seem significantly unchanged (77). Additional synthesis during the recovery period between the end of the temperature treatment and the first synchronous division poses the interesting problem of whether this additional synthesis of both nucleic acids is necessary for the subsequent

division. If it is, the results would agree with findings on division-linked DNA synthesis (37), and in changes of acid-soluble RNA prior to synchronous division in *E. coli* (90).

Unfortunately, all of the biochemical analyses reported can only be expressed as per average cell, and nothing can be inferred about the synthesis in the individual cells which were randomly distributed over the normal life cycle when first disturbed by the temperature shock. Do these cells retain their characteristic synthetic pattern, or is the synthesis in the cell blocked or retarded at a certain stage of its life cycle? In other words, do the cells keep their relative position in the cycle or do they accumulate? Cytospectrophotometric analyses of the Feulgen-stained macronuclei of *Tetrahymena* were conducted in the author's laboratory and in Japan by Mita. Considering that two different strains were used, GL (44, 84) and W (54), respectively, the results are in good agreement. It appears that the relative amount of DNA in the cells (i.e., the amount of DNA in each cell, divided by the average amount for the sample) is relatively constant. Since the average amount increases when treatment is started, continued synthesis in all cells is indicated irrespective of the amount of DNA present in each cell. The results do not favor the idea of a block for DNA synthesis at a specific step in the cell cycle which would have resulted in an accumulated mass culture of cells with a DNA content characteristic for that particular stage. A comparison of DNA content with the average nuclear volume shows that the nuclear volume increased more during the treatment than did the DNA content. If the ratio is established as equal to 1.0 for the normal cells, the DNA-to-nuclear volume ratio gradually decreases with time and reaches a minimum value of 0.56 one hour after treatment, and almost doubles within half an hour prior to fission (84). Such changes might have a definite meaning in relation to structural changes occurring in the nucleus, both during the temperature cycles and prior to cell division.

NUCLEOTIDES, PHOSPHATES

An interesting report on changes in the nucleotide triphosphate content in synchronized cells was made recently by Plesner (66, 67). *Tetrahymena pyriformis* GL grown in mass cultures were sampled at various characteristic growth stages, and acetone powders prepared. Total amounts of nucleoside triphosphate (NTP) were determined by enzymatic phosphorylation of 3-phospho-glyceraldehyde. The normal, average cell of the exponential phase of growth contains 25.3×10^{-6} μ Moles of NTP. This value increases 2.7 times during both the heat treatment and the recovery period, until 30 min. prior to the first synchronous division. Within the following 20 min., the nucleoside triphosphate concentration has sharply increased to almost 15 times that found in the average cell prior to treatment. The amount of ATP, when expressed on a relative basis of dry weight, increased almost four times during the temperature treatment (114). Determination of ATP with the luciferin-luciferase assay could not confirm such an increase of ATP (86), but

showed a significant decrease during the temperature treatment. Reasons for this discrepancy are under study (86).

The total acid-soluble phosphates, including the temperature labile phosphate of the average log cell, increased 1.5 to 2 times during the treatment (84). From the end of the treatment (6.5 hr.) to the onset of synchronous division (7.8 hr.), the soluble phosphates increased 28 to 33 per cent. Acid-insoluble, heat-labile phosphates (which were found to be approximately five per cent of the soluble phosphates) did not change in amount during the treatment when expressed on an average cell basis. However, within an hour prior to synchronous division, the residual phosphates increased (84). A possible interpretation of these findings will be given in the last section.

The phosphate metabolism in synchronously dividing *Corynebacterium diphtheriae* was recently studied (72). The authors concluded from their work that polyphosphate accumulation occurs immediately before periods of increasing cell numbers. These changes in the polyphosphate content are suggested as being physiological events related to cell division.

The application of α -, X-, and γ -radiation, triethylene melamine, and nitrogen mustard to *Saccharomyces cerevisiae* were found not to affect growth but, rather, to inhibit cell division (32, 33). All of these diverse inhibitors produced a uniform pattern in the metabolism of phosphorus. Orthophosphates, acid-insoluble polyphosphates (with 60 to 80 monomers per chain) and DNA phosphate fractions decreased with a concurrent increase in the residual labile phosphate fraction (probably containing ATP, ADP, triphosphate, trimetaphosphate, tetraphosphate, deoxynucleoside diphosphate, and deoxynucleoside triphosphate). No changes were found in the lipid and RNA phosphate fractions. The authors conclude that the cell division inhibitors studied cause metabolic aberrations in the synthesis of DNA and insoluble polyphosphates. That polyphosphates are involved in the budding process of *S. cerevisiae* is evident from incorporation studies of P^{32} into various fractions of x-ray-treated cells, dividing synchronously (93).

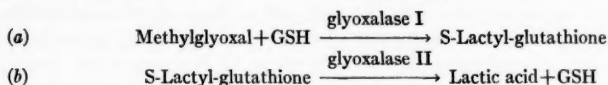
CARBOHYDRATES

The glycogen content of *Tetrahymena*, sampled from the early stationary phase of growth, was found to be 16 to 25 per cent of the dry weight (48). This was confirmed and extended in the author's laboratory (82) on *Tetrahymena pyriformis* GL, grown on a glucose-free medium. After two days of growth, when the cells entered the maximum stationary phase, the glycogen content was found to be 12 to 15 per cent of the lyophilized dry powder, whereas during the exponential phase the glycogen content was only 1 to 2 per cent of dry weight. After the exposure of such a culture to the synchrony-inducing treatment for 6.5 hr., however, the glycogen content rose to levels comparable to the amounts found in cells of the maximum stationary phase. These experiments (48, 82) suggest that inhibition of cellular division in the maximum stationary phase and during synchrony induction might partially be due to an imbalance of synthesis and the utilization of high polymer car-

bohydrates. This view finds support in the work of Kamiya, who recently reported that the concentrations of hexose and pentose increase in *T. pyriformis* W during the synchrony-inducing temperature treatment (31).

SULFHYDRYLS

Rapkin's classical observations of rhythmic changes of acid-soluble SH in synchronously dividing sea urchin eggs have stimulated further analyses (53, 60). Mazia extended Rapkin's attractive hypothesis and emphasized the structural aspects of such oxidation-reduction reactions in connection with the formation and breakdown of the mitotic apparatus and changes in the cellular cortex (53). While Rapkin's report on SH changes has drawn wide attention, another finding of his has apparently been completely overlooked. In the same paper he reported an increase in lactic acid, concurrent with the observed increase of acid-soluble SH (70). If this finding can be verified and perhaps extended, Mazia's structural hypothesis could then be supplemented by a metabolic hypothesis explaining the changes in soluble SH. In many cell systems an enzymatic reaction, involving the conversion of methyl glyoxal to lactic acid has been found (69):



Generally, D-lactic acid is formed in this reaction and L-lactic acid is the glycolytic product. (In some lactic acid bacteria, however, it is the D-isomer that results from the utilization of glucose.) It seems conceivable, therefore, that a simultaneous determination of lactic acid (the dextrorotary species) and glutathione (GSH) might enable us to assign to the often observed changes in glutathione a metabolic role in the "life history" of a cell; this, in addition to its suggested structural function (53).

The concentration of acid-soluble SH in normal and synchronized cells was studied independently in the author's laboratory (83) and by Sugimura, Ono & Mita (94). The latter group used amperometric titrations for the estimation of acid-soluble SH in *Tetrahymena* W and found an approximate 50 per cent increase per average cell during the temperature treatment. A fluctuation in the concentration of acid-soluble SH was claimed to occur in correlation with the first synchronous division step. Our data, on *T. pyriformis* GL, using the nitroprusside test, suggests an approximate fourfold increase per average cell in acid-soluble SH during synchrony-inducing temperature treatments. In the recovery period prior to fission, a further increase was observed. Cytological studies with Bennet's mercury orange stain support the author's findings on a qualitative basis. No convincing explanation of these different results seems possible at the present.

ENZYMATIC STUDIES

Cyclic variations in the peptidase and proteinase activity in synchronously budding yeast were reported recently (98, 99, 100). A pentaploid

strain of *S. cerevisiae* was starved for 3 hr. in potassium succinate medium. During this starvation phase, the protein content per cell remained high, but a significant drop in dipeptidase activity was observed. After the starvation period was completed, the culture was transferred to a rich medium of yeast extract and dextrose and a brief division lag of approximately 45 min. occurred, followed by one or more synchronous division cycles. Dipeptidase activity does increase during this lag, reaching a maximum level immediately prior to the onset of the division step at the inflection point from low to high multiplication rate; this is followed by a marked decline in activity during fission—from 1/7th to 1/9th of the peak activity. The rhythmic variation in enzymatic activity can be traced through the subsequent division cycles. A similar, but less pronounced, cyclic activity was also noted in proteinase. These cycles of increased proteolysis seem to be linked to certain steps preparatory to division. The authors (100) advance the idea that "proteinases and peptidases may be operative in the intracellular reactions aiming at the replenishment of the metabolic amino acid pool necessary for protein synthesis." In their nutritional studies with *S. cerevisiae*, Thorell and Tobias found that the generation time is independent of the concentration of the nutrients over a 100-fold change. On the basis of their studies, they postulate two compartments in the cell: (a) a storage compartment which may accumulate nutrients sufficient for several cell divisions under favorable conditions; the rate of this process should be independent of the presence of oxygen; and (b) a replication compartment which exchanges with the storage compartment and which does not need to draw on external medium; the replication process should be accelerated by the presence of oxygen. This concept could explain nicely the synchrony of division of *Tetrahymena* in inorganic media (19). The chemical nature of the replication compartment (100) may resemble at least a part of the channel of metabolic events through which the normal cell, between two subsequent divisions, passes. (This is outlined in the scheme in Figure 1.)

SYNCHRONOUS DIVISION AND THE NORMAL CELL CYCLE

From the forgoing examples, it becomes obvious that an uncritical attempt to interpret data on changes, in concentration, or in rate of synthesis of any cellular constituent (such as nucleic acid or phosphorus during induced synchrony) as being "normal growth," leads to erroneous concepts about the metabolic events in a "normal" cell. Such a consideration might have been the reason for the development of synchronized systems which resemble more closely the normal life cycle of the individual cell. For example, in an ideal situation we should be able to collect rapidly a large number of cells of the same age from a microbial, steady-state culture. Upon reincubation, these cells should then be in true metabolic phase with each other, the growth-division-equilibrium being undisturbed. The sole handicap and major source of disappointment is, of course, the strong metabolic randomizing tendencies generally found in microbial cultures, as expressed by the large variation in generation times of the individual cells.

Simple procedures for the collection of microbial cells in comparable stages of their life cycles have been developed. These methods are discussed elsewhere (9, 46, 114). One of the best methods involves a fractional filtration of bacterial cells through a filter paper pile (50, 52). The larger cells are held in the top layers of the filter paper and only the smaller cells pass through and can be collected from the filtrate by centrifugation. Upon reincubation of these cells in suitable medium, a brief division lag precedes a synchronous fission wave, during which 50 to 60 per cent of the cells divide. Nucleic acids, and total and protein nitrogen were determined prior to and during such filtration-induced synchrony (50, 51).

A good criterion for judging the "normality" of such a system would be the recurrence of a characteristic synthetic pattern related to the life cycle of the individual cell. Unfortunately, this is not the case for protein nitrogen and RNA in Marujama's filtration system; after the collection of the small cells the rates of protein and RNA synthesis are noticeably lower than during the comparable second postdivision period. Maaløe has suggested that this difference may be due to a brief starvation and anaerobiosis occurring during the isolation procedure (46). Marujama and Yanagita's technique was slightly modified by Abbo & Pardee to eliminate the drawbacks of anaerobiosis and starvation during isolation (1). These authors reported successful synchrony through two to three successive generations in mass cultures of *E. coli* B. In this system the synthesis of DNA, RNA, protein, and inducible β -galactosidase proceeds throughout the division cycle, and also during the process of fission in a constant exponential pattern. Abbo & Pardee consider that these results represent the true time course for the synthesis of these macromolecules within the individual bacteria in its normal life cycle (1).

The induction of synchrony in bacterial mass cultures by mechanical means such as the above fractional filtration which, supposedly, should have no effect on cellular metabolism, might provide us with suitable material for the biochemical study of the normal cellular life cycle. However, all of our biochemical data obtained in this way still has to be expressed per average cell, thus concealing a possible variation of synthetic rates between the individual cells. Therefore, a comparison of the available data on mass synchrony with the results on the synthetic events in single cells is desirable.

Valuable single-cell analyses on yeast (57, 58) and ciliates (35, 36, 107) have recently been reported. Unfortunately, such data are not yet available on the same cells which have been synchronized in mass cultures. Mitchison followed growth in volume and dry mass of single cells of *Schizosaccharomyces pombe* (57) and *Saccharomyces cerevisiae* (58) and found that the growth in dry mass was linear for each budding cycle. On the basis of these findings, he advances the hypothesis that "synthesis is controlled by centers or particles, perhaps microsomal, which remain constant in interphase until division, when they double and partition themselves between two daughter cells" (58). On the other hand, Kimball *et al.* (36) report single-cell growth studies in *Paramecium aurelia*, and from the exponential increase in dry mass during interphase, these authors infer that the synthetic centers (microsomes) are

duplicated during this period at the same rate as the cellular protein. A differential effect on the rate of growth in mass and of division was observed upon transfer of starved, small paramecia into a rich medium. After a short lag period, growth is resumed at the normal rate, but the first division is considerably delayed. The result is an increase in cell size, characteristic of normal multiplication. A shift from rich to poor medium, however, results in retarded growth while division is only slightly delayed. The cells entering fission are therefore smaller than during the steady-state of optimum conditions. It is very likely that a "variation" on the basic "theme" (i.e., reduplication between two subsequent fissions) is found in different systems: the dry weight increase may be linear in *Saccharomyces* as mentioned in the paragraph above, or exponential as in *Paramecium*. It shall be recalled that single nutritional shifts in mass cultures of *Salmonella* also produce a differential effect on growth and division similar to the response of single paramecia to nutritional changes.

SUMMARY AND CONCLUDING REMARKS ON THE PROPOSED CHEMICAL NATURE OF A CHANNEL PREPARATORY FOR DIVISION

Any attempt to interpret separately the data on synchronized systems can, at best, be only fragmentary due to the extreme complexity of the problem and to our ignorance of its details. A comparative analysis of the effect of environmental changes which induce synchrony of division, is, therefore, a tempting approach, although we have to consider the possibilities that such diverse systems as protozoa, molds, and bacteria might attain the same effects—synchrony of division—by quite diverse means. Cautioned by this possibility, we shall try to integrate important findings in cell synchrony and outline some biochemical events necessary to the division of a normal cell. In doing so, we necessarily commit ourselves in a highly speculative manner, justified only by the hope of gaining insight into the channel preparatory to division. A few facts and speculations on which we will build such a "mitogenic channel" will be discussed briefly.

A large body of evidence indicates that the duration of the generation time is more readily affected by environmental changes than is the size of a microbial cell (25, 95). This is shown in synchronization experiments in which temperature shifts or shock are used as an inducing agent. For example, the intermittent heat treatment of *Tetrahymena* suppresses cell division completely, while total growth in the mass culture is reduced to 65 per cent of that of the normal control (75). It becomes obvious that a temperature-sensitive system must be somehow involved. From kinetic studies the author has concluded that heat and cold act in the same way by affecting a denaturation equilibrium of a temperature-sensitive enzyme system (76). This view seems to be supported by Zeuthen's suggestion that high and low temperatures denature sensitive structures by different physical means: "Cold might cause overbonding of a structure and heat might disrupt enough weak bonds to make a structure useless" (114).

Fortunately, a very significant contribution has recently been made, from which one may infer the possible nature of a temperature-sensitive system (13, 14). In six strains of *Tetrahymena* a DPNH oxidase has been discovered that was completely inhibited by mild heating for 10 min. at 40°C. But, under identical conditions, lactic oxidase showed only a 6 to 10 per cent decrease in activity. An exposure to 2°C. for 5 hrs. produces a loss of 80 per cent of the DPNH oxidase activity, whereas only 0 to 10 per cent of lactic oxidase is destroyed. Although no direct analysis of DPNH oxidase activity, occurring during synchronous multiplication, has as yet been made, this enzyme appears to be the best candidate as a temperature-sensitive regulator. Doubts have been expressed by Swann as to whether the control of cell division in terms of a single key mechanism is a useful concept (96); it is certainly in the nature of any such proposal that a mitogenic channel is a highly complex one, but this should not discourage us from looking for the rate-controlling key systems in cellular metabolism. Krebs has shown, for example, that hexokinase acts as a regulator of glycolysis and points out that such systems, being highly vulnerable to extraneous agents, deserve our attention (38). Such a controlling mechanism need not necessarily be the terminal step in the preparation for division. For example, a partial or complete block of the DPNH oxidase activity could result in an accumulation of the substrate, which might then be utilized by the cell in some other, unblocked pathways. Such a differential temperature sensitivity of DPNH oxidase and lactic oxidase could conceivably be one reason for unbalanced growth.

In *Tetrahymena* it has been observed that during the recovery period between the end of the temperature treatment and the first synchronous division, adequate aeration is important for successful synchrony (80). This observation points to the importance of oxygen in the mitogenic channel for this organism (N.B.#2 in Figure 1). It is also known that in the early stationary phase of growth when cell division has ceased, apparently due to the lack of oxygen, the cells are larger than those cells of the exponential phase of growth (62) and a considerable accumulation of glycogen occurs under these conditions (48). A similar increase of cellular glycogen has been found during temperature prior-treatment, when cell division is inhibited (82). This observation of increased glycogen content in cells which are inhibited in their division raises the question as to the possible causal relationship between these two phenomena. It is generally agreed that the main effect of a decreased partial pressure of oxygen causes a decreased rate of formation of ATP and of the oxidation of pyruvate. Pyruvate could then be utilized for glyconeogenesis. The formation of pyruvate involves deamination of amino acids. It is known that *Tetrahymena* produces ammonia (34) and recently it has been observed that the ATP level decreases significantly during heat treatment (86).

The suggested relationship between inhibition of cell division and the accumulation of glycogen is outlined in Figure 1. Any inhibition of the oxidative pathway (by heat shocks at #1 or insufficient oxygen supply at

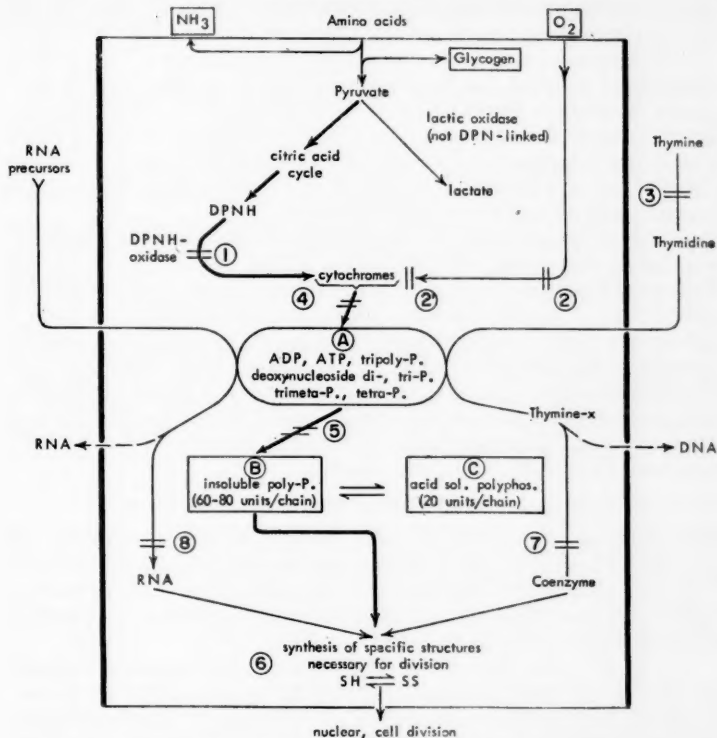


FIG. 1. POSSIBLE CONTROLLING SITES IN A HYPOTHETICAL MITOGENIC CHANNEL

1. Temperature-sensitive system (13, 14); 2. Oxygen-sensitive system (80); 2'. Site of inhibition by carbon monoxide and ether (95); 3. Specific thymine requirement (2); 4. Any inhibition at sites 1, 2, 2', or 3 affects size of fraction A; 5. Radiation-sensitive system, site of chemical inhibition (33, 114); 6. —SH cycle, site of chemical inhibition (16); 7. Formation of a "thymine-containing coenzyme" inhibited by analogues (68); 8. Specific RNA requirement for cell division (60, 105).

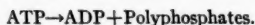
A. Residual labile phosphate fraction (33); B. Insoluble polyphosphate, possible site of control of cation availability (32, 33, 72, 93); C. Acid-soluble polyphosphates, metabolically inert (33). Numbers in parenthesis refer to the literature; see further explanation in the text.

#2) might lead to a decrease of ATP in pool A, with a simultaneous accumulation of glycogen and the formation of ammonia.

Many papers presenting facts and ideas on the energy metabolism of growing and dividing cells have been published. Recently, Wilkinson dis-

cussed the problem of energy storage compounds in bacteria (108). Actually, for quite some time, various cellular phosphate fractions have been looked for which might play a specific role in a cell's preparation for division. Based on studies of the effect of carbon monoxide and ether on synchronously dividing sea urchin eggs, Swann proposed the hypothesis of a "continuous filling energy reservoir during interphase." The energy siphons out when a certain level is reached, triggering division (95). This hypothesis has been adapted and modified by Scherbaum (76) and later by Zeuthen (114) for a speculative explanation of cell synchrony in *Tetrahymena*.

Recently, the connection of cell division inhibition and the phosphorus metabolism of growing yeast cultures was studied by Katchman *et al.* (33). Various inhibitors, such as α - and γ -irradiation and x-ray, nitrogen mustard, and triethylene melamine produce the same biological effect: inhibition of cell division without a concomitant inhibition of growth in mass, resulting in the formulation of paired enlarged yeast cells. A uniform response in these cells was also found in the metabolism of phosphorus. A decrease in insoluble polyphosphates and an increase in residual labile phosphorus fractions were found to occur. This result was interpreted by Katchman *et al.* as being a postulated enzymatic block in the reaction,



Direct evidence for the role of insoluble polyphosphates in synchronized cell division of *Corynebacterium diphtheriae* was recently provided by Sall *et al.* (72). Since such detailed studies of the phosphorus metabolism in synchronized *Tetrahymena* is not yet available, the results on bacteria were utilized for the metabolic scheme in Figure 1.

In Figure 1 three phosphorus fractions have been considered; namely (A) the residual labile phosphate (ATP, ADP, tripolyphosphate, trimetaphosphate, tetraphosphate, deoxynucleoside diphosphate and deoxynucleoside triphosphate); (B) acid-insoluble polyphosphates comprising 60 to 80 monomere units per chain; (C) acid-soluble polyphosphates comprising 20 units per chain. Which, if any, of these fractions or parts thereof, would fit into the reservoir concept cannot be answered at the present. Unfortunately, the metabolically-active, acid-insoluble polyphosphorus changes considerably during the steady-state of exponentially growing *S. cerevisiae* (32). Aside from a possible source of energy in the "energy pool hypothesis," these polyphosphates might have a control function by their ability to inactivate enzymes or to form complexes with proteins necessary in intermediate metabolism. This has been proposed by Lindegren (41). Katchman & Fetty consider the possibility that polyphosphates form complexes with monovalent and polyvalent cations (32).

The synthesis of both nucleic acids during the recovery period in the already oversized *Tetrahymena*, suggests a possible role of both nucleic acids in the mitogenic channel (77, 84), as illustrated in Figure 1. Many indications for such a role could be cited, but direct evidence for synchronized cultures is still lacking. Only one illustrative example of the relationship of nucleic acid

metabolism to cell division will be given. Inhibition of cell division by proflavin or tryptaflavin in *Candida albicans* induces elongation into mycelia, and the RNA content of the cells is lowered (60). It is conceivable that a particular RNA fraction is involved in a particular type of protein synthesis required for cell division.

The role of DNA in the mechanism producing synchrony is as enigmatic as is the role of RNA. The definite requirement of thymine in a thymine-less mutant of *E. coli* (2) and the connection between DNA synthesis and cell division in single-shift experiments (37), lead us to the assumption that a specific fraction of DNA or a thymine-containing coenzyme is important in division. A possible role of a thymine-containing coenzyme in cell division has been suggested by Prusoff on the basis of inhibitor studies in *Streptococcus faecalis* (68).

The last step in the postulated mitogenic channel consists of the synthesis of the specific structures for the division process proper. Whether this process is a synthesis *de novo* or only a remodeling of structures already present, cannot be decided as yet. The interesting finding of a significant increase in dipeptidase activity in *S. cerevisiae* at the onset of synchronous budding might be pertinent to this problem (100).

It also seems feasible that specific enzyme systems are available for the local breakdown of cell wall material prior to bud formation in yeast. Falcone & Nickerson have, in fact, studied such a system, comparing the activity of a protein disulfide reductase in a normal and division-less mutant of *Candida albicans*. They found a considerable reduction in the activity of this enzyme in the mutant and concluded that cell division in yeast begins with the reduction of disulfide bonds in the yeast cell wall (16).

A hypothetical explanation of synchrony in *Tetrahymena* can be attempted in the light of a "reservoir concept," as outlined in Figure 1. First, during the interphase of a normal cell, a reservoir has to be filled to a certain level by reactions within the mitogenic channel. The nature of this pool is not as yet known; it might be, e.g., the insoluble polyphosphate fraction (#5 in Fig. 1). At a certain concentration in this pool and, together with other fulfilled requirements (probably the presence of soluble RNA and a thymine-containing coenzyme), a new chain of events might be triggered, leading to nuclear and cytoplasmic division: the synthesis of specific structures necessary for division or remodeling of already-present structures.

Second, a single temperature shock produces an inactivation of DPNH oxidase at Step One in all cells of the culture and a partial depletion of pool #5 in some of the cells, depending on their position in the cell cycle; the older the cell, the more is removed, thus equalizing the pool size in some of the cells. This has already been concluded from studies in which single cells of different ages were exposed to temperature shock treatments (77). Upon release from the shock, reactivation at Step One takes place and pool #5 is replenished, resulting in partial synchrony during which 25 to 35 per cent of the cells in the mass culture divide simultaneously.

Third, the intermittent temperature prior-treatment produces (a) an

inhibition of the temperature-sensitive Step One and (b) a complete depletion of pool #5, the contents of which are channeled into growth processes. With this channelizing effect, we are able to explain the observation that larger cells grow more during the treatment, than do the smaller ones (since the larger cells possess a larger pool) (76). (c) At the same time all of the machinery of the mitogenic channel is enlarged in an inactive state in proportion to the overall increase in protein material. Upon release from the heat treatment, a characteristic recovery period occurs prior to the first synchronous burst of cell division. During this period, the enlarged machinery of the mitogenic channel is reactivated and fills pool #5, which has been completely emptied in all cells at an increased rate. This increased rate of filling the pool actually works against the strong "randomizing tendencies" of the metabolic machinery in normal cells.

The foregoing discussion of the proposed mitogenic channel leaves us with more unresolved questions than we can answer in the light of our present knowledge. The value of this hypothesis may be questioned unless it stimulates further discussion and experimentation. This is the sincere hope of the author.

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GENE ACTION^{1,2}

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Evidence supporting the one gene-one protein hypothesis has increased rapidly in the past ten years. The development of techniques for the analysis of the amino acid sequences of proteins and protein fragments (1, 2) and mutational site sequences in genes (3, 4, 5) has opened the door to a direct experimental examination of the details of this relationship. In addition, the presentation of the Watson-Crick model of DNA (6) and the theoretical explorations of nucleotide coding (7 to 11) have provided a comforting theoretical background to questions of how the genetic material specifies the information essential for the assembling of the characteristic amino acid sequence of each protein. The experimental analysis of the precise mechanism of cytoplasmic protein synthesis has led to an understanding of several major aspects of this process (see review by Novelli in this volume).

The previous history of the relationship of gene-to-protein has been reviewed in detail in recent papers (12, 13), and a list of some 50-odd examples of the effects of mutations on enzymes in 11 different organisms has been published within the last year (13). Therefore, the present paper will be confined to a discussion of studies with well-analyzed gene-protein systems, and systems which show considerable promise for future investigation. Such studies are providing the gross information which will ultimately seek explanation at the nucleotide and amino acid level. The discussion will be restricted for the most part to microbial material. However, the mammalian hemoglobin system will also be discussed since it is proving to be a model for expectations and predictions of the effects of mutations on specific proteins. No attempt has been made to cover every paper on each subject but only those pertinent to the points discussed. In areas related to the main topics considered, general review articles are given as references.

The word "gene" assumes different meanings according to the operations used in characterizing it (3). In this review the terms "gene" or "locus" will refer to a segment of the genetic material carrying the information for the

¹ The survey of the literature pertaining to this review was concluded in February, 1960.

² The following abbreviations will be used: CRM (cross-reacting material); DNA (deoxyribonucleic acid); RNA (ribonucleic acid); TPN (triphosphopyridine nucleotide).

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amino acid sequence of a single protein (or of a single polypeptide chain if the protein is composed of more than one chain). It is considered that there are many mutational sites (3, 4) within such a unit and that recombination of any kind may occur between these sites. The term "allele" will be used to designate different forms of a gene and mutationally altered forms of independent origin. It is realized that these definitions represent a hope rather than a reality and are imprecise for cases in which all the polypeptide chains of a protein are controlled by adjacent or contiguous parts of the genetic material.

Current concepts.—On the basis of experimental findings and theoretical considerations, a working hypothesis of the relationship of gene to enzyme has been derived (14). It is assumed that the complementarity of DNA (6) provides a mechanism for the exact replication of DNA by the DNA-synthesizing system discovered by Kornberg and his associates (15). It is further assumed that there are specific base sequences in DNA corresponding to each amino acid and that the order of these base sequences determines the order of amino acids in specific proteins (14). A point mutation is considered to represent a change of a single nucleotide in a DNA strand (6). Thus, the DNA segment corresponding to a protein would have a large number of potential sites at which mutational changes could occur (3, 4, 5) and probably more than one per amino acid. Non-reversible mutations would represent changes of more than one nucleotide pair or losses of one or more base pairs. The information carried in the nucleotide sequence of DNA is presumably transferred to a corresponding nucleotide sequence in RNA, and the soluble RNA-amino acid complexes pair with this RNA at specific sites (16). The amino acids are thus properly oriented and a protein molecule can be synthesized. Little information is available concerning the problem of folding of polypeptide chains, but it has been argued that the amino acid sequence itself determines the folding observed in the native protein (14).

Coding.—The coding of the amino acid sequences of proteins by the nucleotide sequences in DNA requires some 20 meaningful combinations of the four bases to account for the common amino acids in proteins. Various codes with 20 meaningful base sequences have been proposed (7, 9, 10, 11). Some of these codes attempt to overcome the difficulties imposed by the overlapping information of adjacent nucleotides by assuming that certain base sequences do not code for amino acids (9). One code has been proposed that deals in a similar manner with the complementary sequences on the second strand of DNA (11). Several of these codes and the coding problem have recently been reviewed elsewhere (17).

From an evolutionary point of view it might be expected that the same or very similar codes would operate in most living organisms. If the code consists of permutations of the four bases, the guanine(G)+cytosine(C)/adenine(A)+thymine(T) ratio in DNA's of all organisms should be similar. Direct experimental examination of DNA's from various bacterial species

has shown that this is not the case: ratios varying from 0.45 to 2.7 have been reported (18, 19, 20). The RNA's of the same organisms varied only slightly in composition (range of $G+C/A+U$ (uracil) of 1.03–1.45) (20). However, until it is known how many copies of different molecular types of RNA are present in a cell, the similarity or difference of base ratios in DNA and RNA cannot be readily interpreted.

The studies of Sueoka *et al.* (21), Marmur & Doty (22), and Rolfe & Meselson (23) indicate that these differences in $G+C/A+T$ ratios could not be due to the presence of a block of non-coding DNA because it was found that there is a fairly uniform distribution of GC pairs throughout the DNA's of many of the organisms with different $G+C/A+T$ ratios. The following are some of the explanations of this dilemma which have been considered (21, 24): (a) The code is different in different organisms; (b) there are "commas of nonsense" separating the nucleotides determining each amino acid, and the composition of these varies from organism to organism; (c) segments of the genetic material (uniformly distributed throughout the DNA of an organism) are not directly concerned with the coding of amino acids, and the amount or composition of this material varies from organism to organism; (d) the amino acid composition of the proteins of different organisms varies appreciably.

A new code has been proposed by Sueoka *et al.* (21) and by Sinsheimer (25), which is consistent with the variations in $G+C/A+T$ ratios detected. The source of information in this code is the sequence of 6-amino and 6-keto groups in the DNA. In this code cytosine would be equivalent to adenine; guanine to thymine, and thus the base ratios could vary considerably from organism to organism without disturbing the coding.

Chemical mutagenesis.—If the alteration of specific DNA bases could be correlated with amino acid changes, it would be possible to decode at least that portion of DNA concerned with amino acid sequence. Although techniques have been developed (2, 26, 27) for the detection of the amino acid changes in a protein associated with mutations, there is as yet little hope of directly analyzing the corresponding base changes in DNA which are presumably responsible for these amino acid changes.

An indirect approach to this problem, employing specific base analogues as mutagens has provided some interesting information on mutagenesis although it has not as yet contributed to the coding problem (28, 29, 30). It has been found that both 5-bromouracil and 2-amino purine produce mutations in phage when phage are grown on bacteria incubated with these compounds (28 to 31). The spectrum of mutations induced in phage with each of these analogues has been determined in great detail and compared with the spectrum obtained following spontaneous mutation (28, 29, 30). In general, the sites affected spontaneously and the sites altered by analogue treatment were different, whereas both analogues gave mutations at a number of common sites. The mutation spectrum following similar treatment with

proflavin was also determined and found to be different (32). The ability of 5-bromouracil and 2-amino purine to induce reversions in spontaneous and analogue-induced mutants was examined and it was found that analogue-induced mutants were generally reverted by either of the analogues, although not always with the same frequency (29). Only a few of the spontaneous mutants responded to either analogue (29).

Bromouracil has been shown to be incorporated into the DNA of phage (33) and bacteria (34) instead of thymine, and to replace a considerable portion of the thymine of an organism without any appreciable heritable effects (33, 34). These facts indicate that bromouracil can function for thymine and that it usually replaces this base specifically. The ability of bromouracil to induce mutations has been interpreted as being due to pairing mistakes of two types: it can replace hydroxymethyl cytosine, although very rarely, in the synthesis of a new strand (incorporation mistake), and it can occasionally pair, when incorporated, with guanine instead of adenine (replication mistake), thus inserting guanine rather than adenine into the new chain (30). The net effect of these two possible mistakes is that bromouracil can bring about the interconversion of an adenine-thymine base pair and a guanine-hydroxymethyl cytosine base pair. The base 2-amino purine presumably acts in much the same way (by replacing adenine), although the available evidence indicates that it is incorporated into DNA poorly, if at all (35). Proflavin is not incorporated into DNA and its mutagenic action has been assumed to result from combination with the phosphate groups of nucleotides during DNA replication (32). This combination presumably results occasionally in the insertion of an incorrect base in a newly synthesized strand. Spontaneous mutations may involve the replacement of a purine by a pyrimidine or vice versa (e.g., adenine^A—thymine^B ↔ hydroxymethylcytosine^A—guanine^B, where the A bases are in one chain and the B bases are in the other) (29, 30). This would account for their non-revertibility by base analogues.

One unexpected observation that has appeared from this work is that there are certain sites which are particularly susceptible to analogue mutagenesis (28, 29, 30). One possible explanation for this finding is that incorporation or replication mistakes are more likely when certain bases are adjacent to the one undergoing change.

It is apparent from these studies of chemical mutagenesis that conditions must be sought where only one of the two possible pairing mistakes can occur, if the information obtained is to be of help in decoding DNA. A mutagen which shows considerable promise in this regard is nitrous acid (36, 37, 38). This compound reacts directly with the purine and pyrimidine bases of nucleic acids, deaminating them, and yielding new bases that may pair differently than the bases from which they were derived (39). Since this treatment can be performed on extracellular phage (37), mutations can be restricted to the variety that involves replication mistakes.

GENE-PROTEIN SYSTEMS

NEUROSPORA CRASSA

Tyrosinase.—Among the earliest studies of gene-enzyme relationships is the analysis of the enzyme tyrosinase by Horowitz and his collaborators. This enzyme offers certain advantages since it is apparently inessential for the vegetative growth of the mold; consequently, strong selective pressure against qualitative or quantitative alterations of enzymatic activity would not be expected (12).

Horowitz *et al.* (40) have reported that four alleles at a locus designated *T* determine different forms of tyrosinase. Genetic studies and detailed analyses of the characteristics of the enzyme produced have been presented for two of the four alleles (41, 42). The Michaelis constants of these two forms of tyrosinase and the ratios of activities with the substrates L-tyrosine and dihydroxy-L-phenylalanine do not differ significantly. The two enzymes are distinguished by a striking difference in stability at 59°C. and are therefore designated thermostable and thermolabile. The energies of activation for the thermal destruction of enzyme activity demonstrate a higher entropy of activation for the labile form. A similar instance of enzyme thermolability (pantothenate synthesis) was among the first cases of mutationally altered proteins in microorganisms (43).

Horowitz and his associates (40) have described two genes which affect the formation of tyrosinase. If a mutant form of either one of these loci is present in the genome, tyrosinase synthesis is diminished or ceases, but formation of the enzyme can be induced by growth on any of a number of aromatic amino acids. The type of enzyme formed under such conditions is controlled by the particular allele of the *T* locus carried in the strain.

Although the primary role in determining the structure of the enzyme resides in the *T* locus, a degree of complexity in the formation and functioning of *Neurospora* tyrosinase is evident from several reports. Kuwana (44) has found that increased tyrosinase activity is obtained if homogenates are prepared from the mycelia of two low-tyrosinase strains which were grown together. Gest & Horowitz (45) have reported that inactivation at 59°C. is greatly influenced by the ionic environment and have analyzed the effect of activators (Na^+ , urea) upon the tyrosinase system. Fox & Burnett (46) have studied the final steps in the formation of the enzyme: the conversion of pro-tyrosinase to tyrosinase by an activating enzyme. It appears likely that the increasing knowledge of the tyrosinase system of *Neurospora* will provide clues to the multigenic control of tyrosinase in *Glomerella* demonstrated by Markert (47) some years ago.

Adenylosuccinase.—In a number of organisms (48 to 51) the terminal step in adenylic acid biosynthesis is the splitting of adenosine monophosphate succinate to adenosine monophosphate. This reaction is catalyzed by the enzyme adenylosuccinase, a deacylase which also catalyzes the biosyntheti-

cally related conversion of 5-amino-4-imidazole (N-succinylcarboxamide) ribotide to 5-amino-4-imidazole carboxamide ribotide (49).

Mutation at the *ad-4* locus of *Neurospora* has been shown by Giles *et al.* (51, 52) to impair the formation of an active adenylosuccinase. Thirty-five mutants at this locus (also called F mutants) were examined in genetic tests; prototrophic progeny were infrequent but were recovered from most of the crosses (53). Some of the mutants have been reported to form qualitatively altered adenylosuccinase with abnormal thermal stability, while others produce an enzyme that differs from the normal in relative activities with the two substrates (53).

Spontaneous or mutagen-induced revertants were recovered from several of the *ad-4* mutants and were examined for adenylosuccinase activity. In each case enzyme activity was detected although the activity in different revertants ranged from 3 to 150 per cent of that of the parental wild-type strain (51, 53). Only a limited number of discrete reversion classes were detected (with respect to enzyme levels) although a different and characteristic reversion pattern was obtained with each mutant (53). At most, three different reversion categories could be distinguished in experiments with any one mutant. In one case, mutant F-12, no revertants were ever obtained with more than 25 per cent of the wild-type adenylosuccinase activity. F-12 revertants which had only 3 per cent of the wild-type adenylosuccinase activity were treated further in order to induce adenine mutations (secondary mutants) (53). In turn, revertants were obtained from these secondary mutants and among 24 reversions examined, none exhibited enzymatic activity greater than 3 per cent of the normal level. It appears likely from these findings that there was extensive mutational damage at the *ad-4* locus in strain F-12. The adenylosuccinase activity of prototrophs produced by crosses of F-12 to a non-reverting *ad-4* mutant was equal to that of wild-type, suggesting that at least a segment of the *ad-4* region in F-12 is unaltered and capable of recombining to yield a fully active "wild type-like" gene and protein.

Complementation has been observed with mutants at the *ad-4* locus (51, 53, 54, 55) (see Complementation).

Glutamic dehydrogenase.—Fincham has partially purified and characterized the L-glutamic dehydrogenase of wild-type *N. crassa* (56, 57). This enzyme catalyzes the formation of glutamic acid from α -ketoglutarate and ammonia and is coenzyme TPN-specific (58).

Eleven independently isolated mutant strains requiring a source of α -amino nitrogen for growth were shown to have less than 0.2 per cent of the glutamic dehydrogenase activity present in wild-type (61). Tests for the presence of inhibitors in the mutant strains were negative, suggesting that the genetic damage resulted in an impairment of the enzyme molecule. The mutant alleles are associated with a single locus, designated the *am* locus.

Pateman & Fincham (59) have examined large numbers of ascospores from crosses between four of the *am* alleles and have shown that wild-type progeny are recovered very rarely or not at all in the populations studied. A

further analysis of the exceptional progeny from one cross in which markers linked to the *am* locus were also segregating gave results not readily interpreted on the basis of either gene conversion or crossing over (59, 60, 61).

Mutation of three of the *am* mutants to α -amino nitrogen independence has been extensively studied by Pateman (62). It is of interest that all the ultraviolet-induced α -amino nitrogen-independent isolates that were recovered represented changes at or extremely close to the *am* locus; no suppressor mutations were observed. All of the revertants possessed some glutamic dehydrogenase activity although spectrophotometric assays of extracts of mycelium from 28 revertants revealed activities of 5 to 100 per cent of normal. The three mutant alleles appeared to differ not only in overall reversion rate but also in the type of reversion most frequently observed (to full, intermediate, or low activity).

One of the revertants, designated 2-1 (61) was isolated from mutant *am*-2 (63). Growth of strain 2-1 on minimal medium was temperature-dependent (63), and it produced a qualitatively altered glutamic dehydrogenase. This glutamic dehydrogenase had little activity at low temperature but could be activated by brief exposure to temperatures of 35° to 50°C.; the activation was completely reversible (57). Incubation of extracts with substrate also gave partial restoration of activity. Similar treatments of wild-type extracts were without effect. The thermal properties of the 2-1 enzyme are not altered during procedures resulting in a 40-fold purification (61). It is suggested that the glutamic dehydrogenase of strain 2-1 can exist in both an active and an inactive form with temperature determining the relative proportions of each (57).

Complementation between certain of the *am* mutants (59, 61, 64) has been observed and is discussed below.

Tryptophan synthetase.—The recent finding (65 to 69) that the tryptophan synthetase of both *Neurospora* and *E. coli* catalyzes three biochemical reactions: (a) indole+L-serine→L-tryptophan; (b) indoleglycerol phosphate+L-serine→L-tryptophan+triose phosphate; (c) indoleglycerol phosphate⇌indole+triose phosphate, has led to the clarification and extension of previous studies of the effects of mutation on tryptophan synthetase formation in these two microorganisms. Evidence has been presented that reaction (b) is the physiologically effective reaction in the formation of tryptophan (66, 67).

Over 100 tryptophan synthetase mutants of *Neurospora* have been described (70, 71, 72), approximately 30 of which have been examined in genetic and biochemical tests (68 to 73). Crosses with these mutants have shown that mutations leading to impairment of tryptophan synthetase formation are all clustered in a very small region of the genome, the *td* locus (71, 72, 74, 75). Mutational site mapping has recently been achieved by Lacy (76) and Bonner (71) and it has been found possible to arrange the mutationally altered sites of a selected group of mutants in a linear order.

In early studies with this enzyme system it was found that most of the

mutants lacking tryptophan synthetase activity in reaction (a) formed a cross-reacting material (CRM) which combined with antibodies to the enzyme (77). Some mutants had neither CRM nor enzyme activity (77) and it was therefore concluded that CRM represented an altered form of the tryptophan synthetase enzyme. It has now been shown that the CRM's produced by four tryptophan synthetase mutants are distinguishable (68, 69, 78, 79, 80). In addition, an altered tryptophan synthetase enzymatically active in reaction (a) alone has been detected (72).

One of the strains, *td-24*, is capable of growth in the absence of tryptophan at high temperatures, and was found to form an altered tryptophan synthetase combined with a dissociable inhibitor (80). By suitable fractionation procedures the inhibitor was removed, yielding a fully active tryptophan synthetase which is, however, much more susceptible to inhibition by heavy metals than is the wild-type enzyme. The ability of this strain to grow without tryptophan at high temperatures has been satisfactorily accounted for by the finding that the Q_{10} of the mutant enzyme between 25° to 37°C. is approximately two to three times higher than that of the normal enzyme (81).

In two *td* alleles (69, 79), the cross-reacting protein could carry out reaction (c) but was ineffective in reactions (a) and (b). With both of these CRM's, pyridoxal phosphate was required for activity, although this coenzyme is not normally involved in reaction (c). It was also shown that hydroxylamine, an effective inhibitor of tryptophan synthetase in reactions (a) and (b) and of other pyridoxal phosphate-requiring enzymes, had no effect on the catalysis of reaction (c) by the CRM from one of these mutants (78, 79). It seems likely, therefore, that the pyridoxal phosphate is not required as a functional component in the reaction but rather for orientation of the enzyme surface for effective catalysis. This conclusion is supported by the finding with the enzyme from one of these mutants (69) that serine was also required for reaction (c). The affinity of the altered enzyme for serine in this reaction was determined (69) and was found to be approximately the same as the affinity of the normal enzyme in reactions (a) and (b).

Two other *td* mutants produced an altered tryptophan synthetase which was effective in reaction (a) but not in the other two reactions (72, 79). In most of the mutants which formed CRM, however, no enzymatic activity in any reaction could be detected (78, 79).

The tryptophan synthetase of *Neurospora* has been highly purified recently by Mohler & Suskind (82, 83) and its molecular weight estimated at 140,000. The *in vitro* increase of tryptophan synthetase activity on incubation of suitably supplemented extracts of *Neurospora* conidia has been described (84). If this increase can be shown to represent true *de novo* synthesis, it will permit additional experimental approaches with this system.

Complementation of certain of the *td* alleles has been observed and the action of suppressors on mutants at the locus has been studied (see below).

ESCHERICHIA COLI

Tryptophan synthetase—*E. coli* tryptophan synthetase differs from the *Neurospora* enzyme in that it is composed of two separable protein components termed A and B. Both protein components are required for the effective catalysis of reactions (a), (b), and (c) although A alone and B alone catalyze reaction (c) and (a), respectively, at low rates (67, 78). Mutations have been detected which affect each of these components separately (85). In addition, one group of mutants has been observed which lacks both component A and component B activity in any of the three reactions and also lacks any CRM related to either of these proteins (85). Genetic tests with these strains indicate that the loci controlling proteins A and B have been deleted (86). These loci are very closely linked in *E. coli* (86) as well as in *Salmonella typhimurium* (87), and are probably adjacent to one another. The mutationally altered sites in many of the various A and B mutants of *E. coli* have been mapped, using transductional techniques, and a linear order has been established for most of the altered sites (78, 85).

An examination of 47 ultraviolet-induced A mutants and 36 ultraviolet-induced B mutants has been carried out (78, 85). Approximately half of the B mutants form a CRM which reacts with antibodies to the B protein and half do not form CRM. Four of the altered B proteins have been examined in enzymatic tests (85) and all are active with normal A in reaction (c) but not in the other two reactions. Furthermore, these altered proteins are approximately as effective in this reaction as is the normal protein.

Sixteen of the A mutants produce a CRM related to the A protein (78, 85). These CRM's are active only in reaction (a) (with normal B), and are approximately as active in this reaction as is the normal A protein. The CRM's of certain A mutants appear to have distinctive properties (85, 88). Mutants of one group form an A protein which is considerably more acid-labile than is the normal protein; a second group forms an A protein which is considerably more heat-labile than is the normal protein. Members of each of these groups map very close to one another or at the same position; the two groups are at opposite ends of the A gene (78, 88).

The A protein from the normal strain is relatively resistant to inactivation by acid treatment which precipitates most *E. coli* proteins. This fact has been employed in the purification of the A protein to a state which is ultracentrifugally and electrophoretically homogeneous (78). In addition, *E. coli* mutants form to 2 per cent of altered component A when grown on low levels of tryptophan; thus, large amounts of material can be obtained for biochemical analysis (78). The A protein has a low molecular weight, approximately 29,500, and this small size makes it a favorable subject for studying amino acid changes associated with mutations (78).

Revertants and partial revertants obtained from CRM-forming and non-CRM-forming A and B point mutants were examined (78, 89), and it was found that in every case reversion restored a protein corresponding to the

protein originally affected, i.e., reversion in A mutants yielded effective A protein and in B mutants, effective B protein. In several instances, immunological and enzymatic tests of partial revertants (strains that grew poorly in the absence of tryptophan) indicated that the restored proteins are somewhat less active enzymatically in at least one of the reactions than is the corresponding normal protein (78, 85).

It is of interest that in both *N. crassa* and *E. coli* similar types of altered tryptophan synthetase proteins have been observed. The one obviously important difference is that in *Neurospora* CRM's are recovered which are enzymatically ineffective in any of the three reactions. To account for this difference it has been suggested (78, 85) that, although the AB combination is required for catalysis of all three reactions in *E. coli*, each of these proteins can carry out one of the reactions (A reaction (c), and B reaction (a) at the normal rate if it is combined with a normal or altered second protein. Provided that mutation has no effect on the ability of A or B to combine with the other component, a normal catalytic rate would be observed in the reaction associated with the unaltered member. Theoretically, it should be possible to obtain altered A's and B's which cannot combine with the other component; these might be detected as enzymatically ineffective CRM's.

Alkaline phosphatase.—The enzyme alkaline phosphatase in *E. coli* is relatively heat-stable and can be purified with ease (90). In addition, growth conditions have been found under which as much as 6 per cent of the total *E. coli* protein is alkaline phosphatase (90). The enzyme has a fairly high molecular weight of 80,000 but various lines of evidence indicate that it may be composed of two identical polypeptide chains of about 300 amino acids each (91, 92).

Alkaline phosphatase-less mutants (ten induced with ultraviolet and three with high-energy electrons) have been isolated and studied (91). Only one mutant has been found to form a protein which is an altered form of the enzyme. A satisfactory linear order for the mutationally altered sites responsible for alkaline phosphatase defects has been established, using Hfr \times F $^-$ crosses and determining the frequency of appearance of alkaline phosphatase-producing recombinants in reciprocal crosses. All the mutationally altered sites are located in a small region of genetic material comprising approximately 0.01 per cent of the total length of the Hfr chromosome. Several of the mutations may be deletions.

Six leaky mutants were also isolated (91); two of these have been mapped and the mutationally altered sites found to lie within the phosphatase gene. Five of these mutants do not form enzyme in the genetic background in which they were isolated (Hfr strain) but produce some enzyme when the mutations are introduced into the F $^-$ strains. This enzyme from F $^-$ cells elutes from a diethylaminoethyl cellulose column at the same position as the normal enzyme (91), suggesting that the detected activity is associated with a similar protein. The small amount of alkaline phosphatase activity in these strains is not associated with a large amount of CRM, indicating that in the

F⁻ strain only small amounts of the alkaline phosphatase protein are formed. However, one of the leaky mutants does form about as much alkaline phosphatase protein as the normal strain but the mutant protein is only 0.3 per cent as active as the normal enzyme. If the enzyme from leaky mutants proves to be indistinguishable from the normal enzyme in amino acid sequence, it would indicate that information relating to the quantitative aspects of enzyme formation is contained in the phosphatase gene.

Revertants have been obtained from all but two of the mutants and a suppressor mutation affecting one of the strains has been detected (91). One revertant was found to produce an altered phosphatase that was less resistant to heat inactivation and somewhat less active enzymatically than was the normal enzyme. Protein degradation studies are being performed with mutant and normal phosphatases and with the phosphatase formed by revertant strains (91, 92).

Galactose enzymes.—Three enzymes, galactokinase, galactose-1-phosphate uridyl transferase, and uridine diphosphogalactose-4-epimerase are involved in the metabolism and conversion of galactose to an intermediate in the glycolytic pathway in *E. coli* (93, 94). Mutants lacking any one of the enzymatic activities but capable of forming the other two enzymes have been obtained (94). In addition, one mutant produces small amounts of all three enzymes, and another forms low levels of two of the enzymes (galactokinase and galactose-1-phosphate uridyl transferase) but not of the third. The biochemical nature of the defect in these strains is not known; it does not appear to be the absence of a galactose-permease, because the formation of β -galactosidase can be induced in these mutants (94).

Genetically this system has many desirable features. Mapping can be performed by crosses or by transduction with phage λ (95, 96, 97). The genes controlling the formation of at least two of the three enzymes appear to be closely linked (94, 97). Under certain conditions, λ transfers genetic material from this *gal* region with high frequency; it does not transfer material from other parts of the genome (95, 96). Complementation between various *gal* mutants can be examined since the *gal* region and closely linked material from one genotype can be introduced by transduction into cells of a different genetic constitution (96, 97). Perhaps the greatest advantage of this material is that the λ phage can be used to select the particular segment of DNA which comprises the *gal* region (95, 98). The recent exciting finding (99) that transformation can be accomplished with DNA prepared from such phage may perhaps lead to the analysis of the DNA corresponding to the galactose enzymes.

β -Galactosidase.—The β -galactosidase of *E. coli* is undoubtedly one of the most thoroughly investigated bacterial enzymes. It has been one of the principal objects of studies of induced enzyme formation (100, 101) and of the problem of protein turnover in growing bacterial populations (102, 103, 104). It can be produced in large quantities and is easily purified (101), and is therefore a likely subject for correlative studies of gene changes and amino

protein originally affected, i.e., reversion in A mutants yielded effective A protein and in B mutants, effective B protein. In several instances, immunological and enzymatic tests of partial revertants (strains that grew poorly in the absence of tryptophan) indicated that the restored proteins are somewhat less active enzymatically in at least one of the reactions than is the corresponding normal protein (78, 85).

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acid sequence studies. This system has the further advantages of ease in detection of mutations leading to loss of an active enzyme, and of manipulation of the level of enzyme formed.

For many years the genetic control of β -galactosidase formation appeared to be very complex (105, 106), but recent studies have permitted reasonable interpretations of some of the major types of changes which have been seen in studies with this system (107). Three closely linked genetic regions are clearly associated with specific aspects of β -galactosidase formation (107). One controls the galactoside "permease," the system which is responsible for maintaining high internal concentrations of inducer. The second region determines constitutiveness versus inducibility. The third appears to be concerned with the β -galactosidase protein itself since it has recently been shown (108) that mutations within this region lead to the production of proteins antigenically similar to β -galactosidase but enzymatically inactive. A linear order for eight mutationally altered sites within this region has been established by appropriate crosses (107). One peculiar mutant recombined freely with all of the other mutants but was linked to the inducibility region when examined by transduction tests. Two of the mutations in the region presumably controlling the structure of β -galactosidase also led to loss of ability to form the permease system—but they are nevertheless not deletions. It is hoped that additional work will provide explanations for these observations.

The recent demonstration that there is a repression mechanism in the β -galactosidase system that keeps the enzyme level low in the absence of inducer is of particular interest (107). It appears that constitutiveness is the absence of a repressing factor which is present in uninduced cells and which inhibits enzyme formation. The ingenious experiments leading to these conclusions were based on the fact that during crosses, genetic material but no cytoplasm is introduced into the recipient cell (107). Therefore, it was possible to examine the effect of the cytoplasm of the recipient upon the functioning of the genetic material introduced from the donor with respect to β -galactosidase formation. It was concluded that inducible cells probably produce some substance (repressor) which prevents the synthesis of β -galactosidase (in the absence of an inducer) while constitutive cells do not. The nature of the repressor and its interaction with inducers, and its site of action remain to be determined. Some recent data suggest that the repressor substance may be RNA (109).

BACTERIOPHAGE PROTEINS

The ease with which fine genetic mapping can be performed with bacteriophage (3, 110, 111) has prompted several investigators to search for suitable phage proteins for comparative studies of mutational changes and alterations of protein structure. Bacteriophage lysozyme is being studied by Koch & Dreyer (112) and Anfinsen (113), and preliminary findings indicate that many properties of the purified material resemble those of egg white lysozyme. The enzyme appears to have a very low and therefore favorable molec-

ular weight. The head, tail sheath, and tail fiber proteins of bacteriophage have also been purified and examined as possible subjects of gene-protein studies (114). Of these, the head protein seems the most amenable to analyses of this type (115) and appears to be composed of a large number of subunits of molecular weight about 80,000 (116).

Some of the relatively simple plant viruses such as tobacco mosaic virus and turnip yellow mosaic virus are also excellent potential materials for studying protein changes although there is no means as yet for mapping mutationally altered sites. Techniques are being developed for determining base sequences in RNA (e.g., 117) so perhaps it will be possible to correlate directly amino acid changes in these viruses with base changes. The recently discovered mutagenic action of nitrous acid on tobacco mosaic virus (118) should prove to be a powerful tool with this material.

The small phages, ϕ X-174 and S-13, are also obvious choices for study in view of their size (119, 120) and presumed simplicity. They are of additional interest because their DNA is in the form of a single rather than a double strand (120, 121). Genetic recombination has been reported in phage S-13 (122).

HUMAN HEMOGLOBINS

Hemoglobin A, a protein which migrates as a single homogeneous electrophoretic component, comprises about 90 per cent of the hemoglobin in the erythrocytes of normal human adults; the remainder consists of various different components (123, 124). Hemoglobin F, a molecule with electrophoretic properties differing from those of A, is the major normal hemoglobin of infant blood but may also be found in varying amounts in adults with certain pathological conditions. In addition, substantial amounts of hemoglobins with mobilities unlike those of A or F may be present in the blood of certain individuals. The electrophoretic studies of Pauling *et al.* (125) and genetic studies of Neel (126) in 1949, established the genetic basis of one such variant hemoglobin. The electrophoretic method for detecting abnormal hemoglobins in both adults and infants has since been widely applied. A recent paper (127) lists some 22 hemoglobin anomalies. The chemical alteration in the hemoglobin molecule that is responsible for the altered electrophoretic behavior has been identified in eight types (Table I), and attention will be devoted mainly to these.

The hemoglobin A molecule has a molecular weight of 66,000 to 67,000 [for summary of data see (128)], and is symmetrical about a dyad axis (129). The molecule contains four heme groups (129) and two pairs of polypeptide chains (130). The chains are distinguished by their N-terminal amino acid sequences: the α chain has the sequence valyl-leucyl and the β chain the sequence valyl-histidyl-leucyl (130 to 133). Upon treatment with mild acid, the molecule dissociates reversibly into two unlike halves consisting of one pair of α chains (α_2) and one pair of β chains (β_2) (134, 135). The isolation of the α_2 and β_2 units has been accomplished by Ingram, who has also studied

the peptides produced by tryptic digestion of each chain (136). One half-molecule of hemoglobin A contains 280 amino acids and the α_2 and β_2 components appear to be approximately equal in size (136). Hemoglobin F also contains two pairs of peptide chains (137); one pair is similar if not identical to the α chain of hemoglobin A (138) but the second peptide chain, designated γ , has glycine as the N-terminal residue (137).

Investigations of the blood of individuals with sickle-cell anemia demonstrated that a variant hemoglobin (hemoglobin S) with electrophoretic mobilities different from A, was present (125). The formation of hemoglobin S is inherited in a Mendelian fashion. Hemoglobin A is absent from the erythrocytes of individuals who are homozygous for the mutant gene. Both hemoglobins, A and S, are produced in heterozygotes and both are present in each red blood cell (128).

The mode of inheritance of hemoglobins C, G, E, and I (Table I) appears to be similar to that described for hemoglobin S. Hemoglobin D probably represents a heterogeneous group of different altered hemoglobins (139); the familial data available for some D types indicate that they are inherited as single gene defects (128, 139).

Studies on the inheritance of the production of the abnormal forms of human hemoglobin, lead to the view that two genes (one determining the structure of the α peptide, and the other determining the β chain) may be concerned with the synthesis of the hemoglobin molecule (128, 136, 140). Mutations in either gene might result in the formation of an altered protein whose electrophoretic behavior reflected a change in charge caused by an amino acid substitution. Smith & Torbert (141) have suggested that two genes control the synthesis of hemoglobin on the basis of studies of a family in which the genes determining sickle-cell hemoglobin and another abnormal hemoglobin known as Hopkins-2, segregated independently. It has recently been shown that these two hemoglobins are, in fact, altered in different chains (136, 142, 143). The possibility that a third gene is implicated in the synthesis of hemoglobin has been reported by Schwartz *et al.* (144) and will be discussed below.

That hemoglobin S does represent a protein with an amino acid sequence different from that of hemoglobin A, was shown by Ingram in 1957 (145). There is a single amino acid difference between A and S, and this difference is represented twice since each chain is present twice in the hemoglobin molecule. Ingram separated the peptides produced by tryptic digestion of heat-denatured hemoglobin by combined electrophoresis and chromatography on filter paper (26, 27). The resulting chromatogram is termed a "fingerprint" of the protein, and the position of peptides from digests of various hemoglobins may be readily compared. In each of the abnormal hemoglobins so far analyzed, only one of the tryptic peptides found in hemoglobin A has been found to be altered (Table I). For four of these, the abnormal peptide has been shown to differ from the normal in a single amino acid (132, 133, 136, 139, 145, 146, 147); in the remaining cases analysis has not proceeded

TABLE I

ALTERATIONS IN THE PRIMARY STRUCTURE OF HUMAN HEMOGLOBIN

Hemo- globin Type	Number of altered peptide in: α chain β chain		Amino acid altered			Reference
			No.*	Amino acid in Hb A	Amino acid substituted	
S		4	6	glutamic	valine	132, 136
C		4	6	glutamic	lysine	132, 136
G		4	7	glutamic	glycine	133, 136
E		26	9	glutamic	lysine	136, 147
D β		26				136, 139
D α	23					136, 139
I	23				tryptophan	136, 148
D γ	number not given but diff. from above					139

* The amino acid residues are numbered from the N-terminal end of the peptide.

this far. The combined evidence demonstrates that alteration of the primary structure of the hemoglobin molecule has occurred in a minimum of five different locations, that alteration in one chain is independent of alterations in the other, and that each alteration seems to affect only one amino acid.

Hunt & Ingram (132, 146) have reported that the alteration in hemoglobin C is in the same amino acid residue of the same peptide as in hemoglobin S. The data of Ingram (136) and of Vinograd *et al.* (142) have shown that the alteration is in the β chain; in fact, the altered peptide 4 probably represents the N-terminal peptide of the β chain. The amino acid sequence of peptide 4 in the normal and the two forms of variant hemoglobin is (132):

Hemoglobin A Val. His. Leu. Thr. Pro. Glu. Glu. Lys.

Hemoglobin S Val. His. Leu. Thr. Pro. Val. Glu. Lys.

Hemoglobin C Val. His. Leu. Thr. Pro. Lys. Glu. Lys.

It would be expected, on the basis of these findings, that hemoglobins C and S should act as alleles in inheritance. Fortunately, both forms occur with high frequency in some populations and considerable evidence supports the conclusion that the genes controlling hemoglobins S and C are indeed allelic (149, 150).

The abnormal hemoglobin G is altered in residue seven of peptide 4—the amino acid adjacent to that altered in hemoglobin C and S. The composition of peptide 4 in hemoglobin G is (133):

Hemoglobin G Val. His. Leu. Thr. Pro. Glu. Gly. Lys.

If there is a specific genic region controlling each chain, the mutational site altered in individuals producing hemoglobin G should be very close to the mutational sites determining the hemoglobin C and S phenotypes. Recom-

bination between the mutant site controlling the formation of hemoglobin S or C and that determining hemoglobin G would be expected to be an extremely rare event. However, the genetic evidence available has led Schwartz *et al.* (144) to suggest that hemoglobins S and G are, in fact, controlled by different genes. The critical case in the pedigree studied is the single son of a union between a father whose blood contained both hemoglobins S and G and a mother with normal hemoglobin. If the two variant forms were controlled by alleles, the son would be expected to have either hemoglobin S or G in his red cells but no evidence for either was found in electrophoretic tests. Further analysis of this family (in which the presence of thalassemia is a complicating factor) would appear of great importance.

Abnormal hemoglobin E has been shown by Hunt & Ingram (146) to have an amino acid substitution in peptide 26, a component of the β chain (136). The limited data available from one family in which both hemoglobins S and E were segregating suggest, but do not prove, that the two types are determined by allelic genes (151).

A hemoglobin with the same electrophoretic mobility as hemoglobin S was described by Itano in 1951 (128) and designated hemoglobin D. Hemoglobin D could be distinguished from S by the solubility of the reduced form. Abnormal hemoglobins with the characteristic properties of D have been reported from many parts of the world [summarized in (139)], but the work of Benzer *et al.* (139) has shown that at least three varieties exist. A comparison of fingerprints of the hemoglobin D of three unrelated individuals demonstrated that each differed in the particular peptide altered (Table I). These findings indicate the need for supplementing electrophoretic characterization of altered hemoglobins by more extensive analyses if studies of the geographic distribution of the various hemoglobins are to be meaningful (139).

One of the three hemoglobin D's investigated, D α , is altered in peptide 23 (139), a constituent of the α chain (136). One other α chain variant has been reported, hemoglobin I. This is also altered in peptide 23 (148). The use of specific staining reactions showed that peptide 23 in hemoglobin I, unlike that in A, contained tryptophan.

In contrast to the generally clear picture which emerges from the studies of variant hemoglobins described above, the condition known as thalassemia appears to be more complicated. In thalassemia the hemoglobin produced does not differ from hemoglobin A electrophoretically; instead, the quantity of hemoglobin A is reduced with consequent anemia. Itano (128) and Ingram & Stretton (152) have suggested that the thalassemias may represent amino acid substitutions which produce no demonstrable charge difference in the molecule. Mutation at a large number of sites in either the α or β chain could be expected to result in thalassemic phenotypes of varying degrees of severity.

COMPLEMENTATION

When two auxotrophic alleles are present in the same cell, restoration of the capacity to grow in the absence of the specific nutrient required by

the mutants may occur. This phenomenon is termed complementation and it appears to be fairly widespread in microorganisms. In *Neurospora* complementation is detected by forming a heterocaryon—a mycelium that contains in a common cytoplasm nuclei of two different types, each carrying a mutant allele of independent origin. In *Salmonella*, one allele is present in the nucleus and the second is introduced by phage-mediated abortive transduction. The formation of an active protein by interaction of certain alleles has been demonstrated in four systems in *Neurospora*: glutamic dehydrogenase (59, 61, 64), adenylosuccinase (51, 54, 55), argininosuccinase (153), and tryptophan synthetase (79, 154). Other instances of complementation in *Neurospora* (155 to 160) and in *Salmonella* (161, 162) have not been so well characterized.

In all of the studies described, only certain alleles at a locus appear capable of complementation, and in no case has more than 25 to 30 per cent of the enzymatic activity of the wild-type been observed. A comparison of the position of mutationally altered sites in a locus as determined by genetic tests with the capacity of the corresponding mutants to complement in heterocaryons, has been presented for the *am* (59, 61), *ad-4* (54), and *td* (71) mutants. In general, it appears that the more distant the mutationally altered sites are from one another on the genetic map, the more likely it is that complementation will occur. However, exceptions are found, most notably in the glutamic dehydrogenase system where it has not been possible to map the *am* mutants in an unambiguous order (59).

Among the experiments performed with glutamic dehydrogenase, studies of the characteristics of the enzymes extracted from heterocaryons of various *am* strains with the *2-l* mutant are of particular interest because of the altered thermal properties of the enzyme produced by *2-l*. The present indications are that in all of the combinations with *2-l*, some "wild type-like" enzyme is produced as well as the enzyme characteristic of *2-l* (59, 61).⁴

Similar studies of the proteins formed by heterocaryons between certain *td* alleles have been reported (79). Enzymatic and immunological analyses were carried out on extracts of heterocaryons formed between several pairs of complementing *td* alleles and, in each case, what appeared to be a mixture of three tryptophan synthetase proteins was detected: CRM's with the enzymatic and immunological characteristics of the CRM of each of the participating members, and physiologically effective tryptophan synthetase (tryptophan synthetase that will catalyze the conversion of indoleglycerol phosphate to tryptophan). Tryptophan-independent heterocaryons were formed only when both partners were capable of forming a CRM (71, 79, 154). Furthermore, every CRM-forming strain was capable of complementing at least one other CRM-former (71, 78, 79). These findings suggest that

⁴ More recent studies with other *am* alleles have shown that the enzymatically effective glutamic dehydrogenase formed in certain heterocaryons is distinguishable from the normal enzyme. J. R. S. Fincham, *J. Gen. Microbiol.*, 21, 600 (1959).

the ability to form CRM is essential for effective complementation. It is of interest that in *Neurospora* only CRM-forming mutants are suppressible (70).

Complementation studies with 123 alleles at the *ad-4* locus of *Neurospora* have been reported by Woodward *et al.* (54). Some 51 of these mutants were capable of heterocaryotic complementation with at least one other allele. These complementing strains comprised both primary and secondary mutants—the latter derived by mutation to the adenine requirement of reverted *ad-4* strains (see above). The patterns of complementation observed when all the possible pair-wise combinations of the 51 alleles were examined, allowed the mutants to be placed in a unique complementation map composed of seven distinct regions. There appeared to be a good correlation between the "distance" separating mutants on the complementation map and the speed of formation of nutritionally independent heterocaryons, the rate of growth of the heterocaryons, and the amount of adenylosuccinase activity present in extracts of the heterocaryons. Comparisons of the complementation pattern of secondary mutants with that of the primary mutant from which they were derived is of interest. For example, the primary mutant F2 does not complement with any other *ad-4* allele. However, almost half of the secondary mutants derived from a revertant of F2 showed complementation with at least one primary allele located in any one of the seven complementation regions. This is particularly surprising since, in some cases, these secondary mutant strains had been subjected to three separate x-ray treatments. These findings may indicate that complementation can occur only between strains with specific types of mutational damage (54).

The finding of *in vitro* complementation among alleles of the *ad-4* locus by Woodward (55) provides an opportunity for additional approaches to the problem. Adenylosuccinase activity as high as 20 per cent of that observed in *in vivo* complementation was detected in *in vitro* experiments.

As has frequently been pointed out (59), there is no known process of nuclear exchange during the somatic phase of the life cycle of *Neurospora*. Furthermore, the possible formation of diploid nuclei has been investigated with negative results (59). Certainly, if either of these possibilities were to explain complementation, they would have to be fairly common to account for the appreciable amount of "wild type-like" enzyme found in certain instances of complementation. It appears more likely that complementation is a cytoplasmic phenomenon involving protein-protein, protein-template, or template-template interactions although it is also conceivable that protein or template is modified by the complementary nucleus (54, 59). Now that *in vitro* complementation has been accomplished (55), perhaps the cellular components required for this process can be investigated.

The simplest interpretation of the mechanism of complementation invokes recombination of dissociable or separately synthesized polypeptide chains of a protein. Although this may occur in certain cases, the fact that there are at least seven complementation groups at the *ad-4* locus (54) and at

least five at the *td* locus (71, 78, 79, 154), makes this possibility unlikely. Another interpretation is that an enzymatically effective dimer or aggregate is formed by the association of enzyme molecules of both defective types. But, if complementation occurred by this mechanism, there is no obvious reason for any relationship between complementation and distances on the mutational site map. Exchange within polypeptide chains or within the template material controlling their formation seem to be more reasonable mechanisms.

It appears important to the eventual solution of this problem that in all of the systems studied many mutant alleles do not complement. Since the majority of these non-complementing types are capable of reversion, they cannot represent deficiencies. However, they may, as in the *td* system, be incapable of forming any CRM-like protein corresponding to the normal enzyme. This may be a clue to the nature of complementation, but unfortunately it depends upon an understanding of the defects in protein formation in CRM-less mutants.

It has been suggested that the upper limit of 25 per cent restoration of normal enzymatic activity observed in complementation is consistent with a recombination model (54, 61). The entities undergoing exchange might be either polypeptide chains or RNA templates, but if a random reciprocal event similar to crossing-over is assumed, the observed maximum restoration of 25 per cent of normal activity is readily accounted for. This mechanism would also yield a doubly defective protein in amounts equal to the active protein formed. If a heterocaryon were made between two mutants producing recognizably different "enzyme-like" proteins, the use of separative procedures might permit the recovery of such doubly defective molecular species.

The validity of expressing activity in terms of wild-type enzyme levels may be questioned, because the amount of specific "enzyme-like" protein (for example, CRM) of each of the two participating members of a heterocaryon may vary. Thus, 25 per cent of the wild-type enzyme activity may actually represent much less than one quarter of the total enzyme-like protein formed. Enzyme levels in microorganisms under conditions of limiting supply of the end product of a biosynthetic pathway are notoriously variable; in fact, more CRM is found in heterocaryons of most *td* mutants than tryptophan synthetase in the wild-type strain (79). Finally, unless RNA template synthesis and activity is independent of the numbers of specific types of nuclei, the degree of complementation should be related to the nuclear ratio in heterocaryons. It would be of interest to determine whether varying nuclear ratios affect the level of enzyme activity.

HYBRID PROTEINS

Whatever the mechanism underlying the phenomenon of complementation may be, it is of interest to inquire whether allelic complementation exists in higher organisms. The diploid state permits the regular occurrence of heterozygosity for two alleles and it would therefore be expected that hybrid

protein molecules (molecules deriving structural information from two alleles) would frequently be found if interactions similar to complementation existed. Clearly, complementation in diploid species is a rare event, for if it were not, the concept of allelism would be even more confused than it is. It should be emphasized that even in microorganisms the majority of alleles are non-complementary. Since, in most of the work on complex loci in diploid organisms only a few mutants have been used, it is not surprising that complementation is not observed. Rather, opposed results have been obtained in three studies in which a sufficient number of alleles were tested to permit any conclusions. Complementation is not reported in studies of 36 mutants of the *white* locus in *Drosophila* (163). However, in a series of 18 mutants at the *lozenge* locus studied by Green & Green (164), two showed complementation with all 16 of the others. In extensive analyses of the *tailless* alleles of the mouse by Dunn and his colleagues (165), almost every allele appeared capable of complementation with any other. It is, of course, difficult to evaluate these findings in terms of primary structural alterations of a protein.

Hybrid serum proteins have been reported in the F_1 from species crosses of birds (166) and recently in the haptoglobin component of human serum (167). The genetic basis of the inheritance of two electrophoretically distinct types of haptoglobin was shown by Smithies & Walker (168, 169). It has been suggested that the complex patterns observed when one of these types is examined by starch gel electrophoresis may represent polymerization products and that the formation of polymers is also responsible for the putative hybrid protein of heterozygotes (170).

The most intensive available studies relevant to the problem of complementation are those concerning the organization of the α and β units in the hemoglobin molecule. The studies of Field & O'Brien (134) demonstrated that carbonmonoxyhemoglobin A from normal adults dissociates reversibly in acid into two half-molecules. Singer & Itano (171) have shown that this dissociation is asymmetric yielding α_2 and β_2 units. In their ingenious experiments, mixtures of hemoglobin A, F, and C, labeled differentially (either in the heme groups or in the polypeptide chains), were dissociated and permitted to recombine. Examination of the resulting products by electrophoresis showed that the dissociation-reassociation process gave rise to new hemoglobin molecules by the random recombination of α_2 and β_2 units.

The analysis of hemoglobin I (148) provided material for a direct test of recombination between hemoglobins altered in the α chain (I) and those altered in the β chain (S and C). Itano & Robinson (172) have reported that hemoglobin A and a doubly abnormal molecule are produced by recombination of hemoglobin I with either S or C. The same authors (173) have studied blood from individuals heterozygous for hemoglobin S (defect in the β chain) and hemoglobin Hopkins-2 (α -peptide defect) and have obtained similar results. Thus, it appears that the formation of the complete hemoglobin

molecule from α_2 and β_2 half-molecules is a random process both *in vivo* and *in vitro*.

These studies lead to the conclusion that the binding between identical chains is greater than that uniting the α_2 and β_2 half-molecules (171). Single α or β chains have not been found *in vivo* nor are they produced in the dissociation experiments (171, 172, 173). As might therefore be expected, no true hybrid proteins of the types $\alpha^A \alpha^I$ or $\beta^A \beta^S$ have been observed. It has been suggested (174) that identical chains may be synthesized simultaneously or in rapid succession on the same template, and fold into relatively indissociable paired configurations before contact with polypeptides of the other type.

Hemoglobin molecules consisting of four chains of a single polypeptide have been seen in adults and in infants. Peptide fingerprints of hemoglobin H (175), as the adult β_4 hemoglobin is called, show no unusual peptides and support the view that the β chains are identical to those of hemoglobin A (176). In the fetal anomaly, the entire hemoglobin may consist of α_4 units or mixtures of hemoglobin F and the α_4 type may be found (177). Children with α_4 hemoglobin do not produce hemoglobin H in later life, and it is therefore possible that the α chain of fetal and adult hemoglobins is under different genetic control (177). Ingram & Stretton (152) have suggested that either there is a non-electrophoretically detectable change in the β chains of hemoglobin H individuals, allowing a preferential polymerization of β chains, or that, in the presence of thalassaemia affecting the α peptide, an excess of β units is produced. The existence of genes acting as general suppressors of hemoglobin synthesis cannot be dismissed (152) and it also appears possible that specific loci control the assembling of the complete hemoglobin molecule.

The present evidence concerning the formation of hybrid proteins in higher organisms is admittedly meager. If further investigations substantiate those reviewed here, it may appear that hybrid proteins seldom occur. The versatility of heterozygotes, which must account for their high incidence in natural populations (178, 179), cannot then be attributed to the creation of a new protein molecule with new specificities; it seems rather that the apparent selective advantage of the heterozygotic cell may depend upon the presence in it of two molecules each with unique properties. It is now generally accepted that the high frequency of the sickle-cell gene in malarial areas is explained by the increased resistance to certain forms of the disease exhibited by individuals heterozygous for hemoglobin S (180). If the lack of hybrid proteins in diploid species should prove to be general, it might be that special mechanisms have evolved to control the assembly of polypeptide chains in a manner preventing interaction.

SUPPRESSORS

The mode of action of suppressors and other modifier genes requires explanation before any final picture of the nature of gene action emerges

It is clear that the term suppressor includes a variety of genes with quite different, and sometimes very complex, metabolic effects (181). Thus, any genetic change alleviating the mutant phenotype of another locus is classified as a suppressor although the mechanisms of relieving the original defect may be most diverse. From the point of view of gene-enzyme relations, the critical questions can be phrased thus: do all suppressors act indirectly in restoring a phenotype approaching the wild-type, or do some suppressors contribute information leading to the synthesis of a normal (or more nearly normal) protein? If the latter is true, how is the repair effected?

The detection of suppressors in *Neurospora* may readily be accomplished by the analysis of asci from appropriate crosses (182). Recombination experiments in *E. coli* K12 have also led to the recognition of suppressors for several loci (183, 184, 185). A second method, developed by Yura (186), used transduction to demonstrate the occurrence of suppressors in a study of purine-requiring auxotrophs of *Salmonella*; the method has since been applied to *E. coli* (89).

There can be little doubt that suppressors may act indirectly in restoring the mutant phenotype. The work of Lein & Lein (187) and of Strauss & Pierog (188) with acetate-requiring mutants of *Neurospora* has shown that suppressors can act by making an alternate pathway more readily available. The same is probably true of suppression of cysteine mutants of *Salmonella* (189). It is not surprising that suppressors of this type are not allele-specific since, indeed, the mechanism employed circumvents rather than repairs the original defect.

A second type of indirect effect concerns inhibition relief and has been revealed in studies with several systems (190, 191). In the case of the suppressor of the temperature-sensitive mutant, *td-24*, in *Neurospora* (192, 193), the analysis is of particular interest because the tryptophan synthetase formed by the suppressed strain could be examined to determine whether it resembled the mutant enzyme, and was sensitive to metal inhibition (80), or whether it represented a new tryptophan synthetase, comparable to the wild-type enzyme. It was found that the enzyme extracted from strain *td-24* and from a strain of *td-24* carrying a suppressor were very similar and equally metal-sensitive (193). Thus, the suppressor of *td-24* does not affect the properties of the protein itself but appears to remove a normal cellular component which inhibits the activity of the enzyme (193).

Suppressor mutations restoring the ability to grow in the absence of tryptophan have been detected in three other *td* mutants of *Neurospora*. Crosses indicated that many of the suppressor mutations occurred at different genetic sites (194). All of the suppressor genes were highly specific, affecting at most only one other mutant of the twenty-odd strains which were tested. In each case, the suppressor gene restored the ability to form an enzymatically effective tryptophan synthetase protein but only small amounts of enzyme activity were detected (relative to the level produced by the normal strain) (70, 192). Immunological tests with tryptophan synthetase prepara-

tions from these suppressed strains indicated that there is less activity in the indole-to-tryptophan reaction per unit of tryptophan synthetase protein than in the wild-type strain (77). Thus, these suppressed mutants appear to form either a mixture of tryptophan synthetase proteins or an altered tryptophan synthetase protein with lower activity in this reaction.

Suppressor mutations have not been detected in CRM-less strains of *Neurospora*; nor do these strains respond to suppressors which affect CRM-formers. In the analysis of the *td* mutants and in several other cases, it has been observed that the growth of wild-type stocks is appreciably retarded when a suppressor gene is incorporated into its genome (73, 189, 195). In no case has this inhibition been overcome by nutritional supplementation (78); therefore, these suppressor genes must have some as yet undiscovered additional effect on metabolism. The extreme allele specificity of suppressor genes has also been noted in studies with inositol (196), histidine (197), and purine mutants (186). One study with *Neurospora* has revealed that a suppressor gene can restore enzymatic activity in two unlinked mutants blocked at different steps in cystathionine metabolism (196, 198). Whether the two very similar reactions concerned are normally catalyzed by one complex protein in this organism has not been determined.

Suppression of *E. coli* tryptophan synthetase mutants appears to differ from suppression of *Neurospora* mutants in that A and B mutants may be suppressed whether or not they form CRM (85). Suppression of either an A- or a B-point mutant is always correlated with the formation of a corresponding effective protein but, in every case as in *Neurospora*, the level of activity is lower than that of the wild-type strain (85). Whenever a CRM was formed by the original mutant strain a protein resembling the CRM of the unsuppressed mutant and, in addition, a small amount of "wild-type-like" activity were detected upon introduction of an effective suppressor into the genome. The A protein from one such suppressed mutant has been separated into two fractions; one is rich in A-CRM and the other in an A protein with "wild-type-like" activity (199). In this case it appears that suppression does not act indirectly at the substrate or inhibitor level but affects the A protein itself.

Although the action of suppressors is undoubtedly indirect in many cases, these observations on the tryptophan synthetase system raise the possibility that some suppressors may permit the formation of an effective amino acid sequence which can substitute for the damaged portion of the enzyme molecule. It seems clear that the suppressors studied do not form an entire tryptophan synthetase protein because of the allelic specificity they exhibit (192, 199). This specificity is more readily accounted for by the supposition that suppressors are specific for the particular amino acid substitution associated with the mutant lesion and somehow participate in the formation of an effective amino acid sequence. The mechanism by which an effective amino acid sequence could be restored is not known; however, in view of the demonstrated effects of complementation between alleles, one possi-

bility is that a similar restoration of activity can be accomplished by the recombination of products of non-allelic genes. Also, possible as suppression mechanisms are minor changes in the amino acid activating and transfer systems (such that, e.g., the activating enzyme that normally couples a specific soluble RNA to glycine, substitutes alanine 1 per cent of the time).

It is interesting to find that suppressor mutations have not appeared in the extensive studies on adenylosuccinase and glutamic dehydrogenase in *Neurospora* (although suppression of adenylosuccinase in *Salmonella* has been reported) (200). If further work fails to demonstrate suppressors for these loci, it would appear that explanations of suppression involving systems common to the formation of all proteins could not be correct. On the other hand, the general inhibitory effect on the wild-type noted in studies with some suppressors may be indicative of the action of suppressors on systems common to the formation of many proteins.

CONCLUSIONS

Further studies of the amino acid sequences of mutant proteins in specific systems will undoubtedly provide answers to such questions as: (a) Is there a direct linear correspondence between mutational sites on a genetic map and the amino acid sequence of a protein? (b) Is specific information for the folding of a protein contained in a gene, or is folding entirely determined by the amino acid sequence? (c) Can alterations in the sequence of its amino acids affect the rate of synthesis of a protein and account for observed quantitative affects? (d) What is the composition of the "wild type-like" product of complementation and is a doubly-defective protein produced? (e) Do suppressor genes alter the primary structure of the proteins they effect?

Many related and equally important problems must be solved, however, before a complete understanding of all aspects of gene function in the complex environment of a growing, developing, evolving organism is achieved. The coding problem is basic to further progress in the determination of nucleotide-amino acid relationships and to a more satisfactory description of genic structure. It appears to be possible that the nucleotide sequences of amino acid-specific, soluble RNA's can be analyzed, and this will certainly contribute to deciphering the code. It is to be hoped that chemical mutagens of extreme specificity will enable the investigator to modify mutational patterns at will and to follow the effects of mutation on amino acid sequence. It may be that as more examples of amino acid substitutions at specific sites in proteins in different individuals or different species accumulate, some aspects of the code will be revealed. Certain proposed codes predict a large class of "nonsense" mutations and the CRM-less mutations warrant additional attention as possible representatives of this class.

It is, of course, obvious that the determination of gene-protein relationships is a function of the types of mutants which are recovered. For this reason, the problem of the so-called "irreparable" mutants of *N. crassa* is of great importance. It has been shown that the majority of spontaneous

mutants are of this class (201). Until the nature of these mutations is better understood, the possibility exists that only certain types of genic activity are represented in the systems which have been studied.

A third category of genic activities involves genic interactions. Studies on the repression of enzyme formation (202, 203), on associated inducer-concentrating systems (204, 205, 206), and on the expression of alternate states (207, 208) will undoubtedly continue to provide information concerning the integration of functions within the cell. Current concepts provide few clues to the nature of position effect (209) or to the mode of action of mutator genes (210, 211, 212), and these subjects warrant considerable additional attention.

Finally, the nature of the gene-protein relationship during development is an important and puzzling aspect of gene action. An enormous body of information accumulated in recent years [see (213) for references] indicates that in higher organisms the simplicity of the gene-protein relationship which typifies microbial systems is replaced by complexity at the protein level; a multitude of functionally similar but detectably different forms of the same protein have been demonstrated in different tissues in the same individual and, in some cases, in the same tissue. It is, of course, possible that the basic relationship of gene to protein is preserved in higher organisms and that macromolecular interactions and regulatory mechanisms not well understood at the present time play a more important role. Progress in the elucidation of the gene-protein relationships in the last few years has been so rapid and the prospects for the near future are so encouraging that it is probable that such more complex aspects of this relationship will soon yield to experimental attack.

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IMMUNOLOGICAL SPECULATIONS¹

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I shall always regard the differentiation between self and not-self as crucial to all immunological theory.

F. M. BURNET

Or, aucune machine n'est capable de choix, sauf selon des critères précis prédéterminés et inscrits dans son programme.

E. DELAVENAY

INTRODUCTION: LA MACHINE A TRADUIRE

In this discussion of recent ideas concerning the mechanism of formation of classical antibodies, the attention is focussed on the structure of a general conceptual framework rather than on particular processes that may represent important links in the stimulus-reaction chains.

The antibody-forming system in an animal is analogous to an electronic translation machine, the parts of which are scattered among other devices in a factory exercising a large variety of functions. In trying to understand the design and structure of this translating machine, we might obtain useful clues by studying parts such as bits of magnetic tape and photographic film. But it would be equally important to analyse the general performance of the machine, in order to deduce what essential operations have to be provided for, and to try to induce the machine to make mistakes that would reveal limitations of its complexity. Having observed that the machine translates a foreign language into English, we might say: The machine produces English, but recognizes only Foreign. We would realize that both English and the foreign language are composed of the same alphabet, and that single letters cannot be the units that are recognized by the machine. We might also feel sure that a sentence cannot be recognized as a whole because the number of possible sentences would surpass any tolerable complexity. We would probably conclude that an important feature of the machine would have to be an ability to recognize single foreign words, and that the mechanism by which it functions must, in some form or other, include the consultation of a Foreign-English dictionary.

In the interesting analysis of this electronic device by Delavenay (21), several other analogies with the antibody-forming system suggest themselves to the imagination. The idea of pre- and postediting is reminiscent of Burnet & Fenner's (8) modified enzyme proposals, whereas the possibility of inserting an interlanguage between entry and final translation reminds one of

¹ The survey of the literature pertaining to this review was concluded in January, 1960.

Monod's (63) suggestion that the antigen might first be recognized by a permease. The polysemic properties of dictionary items are analogous to Talmage's (101) restrictions on immunological specificity. Even the average length of words, the number of letters available for their composition, and the size of an adequate dictionary seem quantitatively close to their immunological counterparts.

In the following, an attempt is made to provide a basis for a more adequate immunological terminology because thought and discussion suffer from the lack of single words for simple immunological concepts. It should be possible to express Burnet's axiom: that an animal can discriminate between "antigenic determinants not represented in its components" and "potential antigenic determinants which are inert because they are the same as determinants already present in its components" (11), in fewer words.

TERMINOLOGY

The suffixes *-lope* and *-type* are used in words denoting certain units of immunological significance. An antigen particle carries several *epitopes* (= surface configurations, single determinants, structural themes, immunogenic elements, haptenic groups, antigenic patterns, specific areas). Hidden epitopes which become immunologically available only after breakage, decomposition, or denaturation of the antigen are *cryptotopes*. An antibody molecule normally carries, apart from its epitopes, two *paratopes* (= combining sites, reactive segments, complementary cavities, specific receptors). Epitopes are potentially *immunogenic* (12) when present on an appropriate carrier: they may induce the formation of paratopes.

The adjective *paratactic* signifies the existence of a complementary spatial correlation between an epitope and a paratope. The stereospecific system is characterized by *parataxis* which is the properly oriented approach of two paratactic partners to each other preceding their linkage by a paratactic bond.

Identical paratopes are materializations of the same *paratype*; identical epitopes belong to the same *epitype*. All paratypes that are paratactic to a single epitope are *related*: they belong to the same paratypic *family*. All epitopes that are paratactic to a single paratype belong to an epitypic family. Any epitope or paratype may belong to several families.² The relation within a family is immunological and does not necessarily coincide with chemical similarities. The paratactic bonds which members of one family can form with their common partner may be of different strengths.

Epitopes that are carried by components of one individual animal are *idiotopes*. The idiotypes to which they belong constitute the *idiom* of the animal. Any epitope not represented in the idiom is a *xenotype*. The paratopes that occur in an animal at a given moment can be said to constitute

² Analogously, the French word *temps* relates the English words *time*, *weather* and *tense*, whereas the English word *time* relates the French words *temps*, *fois* and *heure*.

its paratypic dictionary. Those paratopes that are present on circulating antibody molecules will normally be paratactic only to *xenotopes*. Whenever useful for the clarity of discussion, a distinction can be made between *para-xenotopes* and *para-idiotopes*. Two animals will have idiotypes in common which thus belong to the idiom of both. The idioms of two individuals of the same species will nearly coincide: each will possess only comparatively few idiotypes that are xenotypes to the other. These individually different epitopes are *allotypes* (73). Epitopes that are common to the idioms of a very large number of species are *pantachotypes*. For practically all animals, *pantachotypes* will, therefore, not be immunogenic. They are included among the class of epitopes because of the idea that protozoa (87) and very simple animals (3) may have found it useful to develop paratypes against pantachotypes as a simple means of attracting a large variety of foreign organic matter.³

NON-SYMMETRY OF PARATACTIC CORRELATION

The spatial correlation between an enzyme and its substrate, between a hormone and its target, between fertilizin and antifertilizin (103), between a virus and its cell receptor, are a few non-immunological examples of the abundant use that nature makes of stereospecific systems. Different classes of configurational elements are probably involved in these different systems (63).

It is conceptually important to examine the question whether or not immunological paratactic correlation is symmetrical: are paratopes epitopes? Experiments designed to settle whether or not the paratopes presented by antibody molecules are potentially immunogenic have been indecisive. If a paratope is a construction *de novo*, as postulated by the direct template theory of antibody formation, then it is probably not an epitope, because it would be a xenotope to the animal itself and, by inducing the formation of paraparatopes, would defeat itself. If, on the other hand, paratopes are present prior to their selection by invading xenotopes, as postulated by all selective theories, they could be idiotopes, and might well be immunogenic in another animal.

Cushing & Campbell (20) have concluded that paratopes are not immunogenic, from experiments showing that chicken antipneumococcus antibodies and rabbit antipneumococcus antibodies do not cross-react when tested as antigens in guinea pigs. This evidence is inconclusive; if paratopes are epitopes, an antipneumococcus paratope might belong to the idiom of all three species.

If paratopes are epitopes, the universe of epitopes could be divided into two classes, either of which could be named paratypes; and any two paratactic partners could be interchanged between the two classes. Accordingly,

³ τόπος = region; τύπος = sign; ἐπί = on; παρά = alongside; κρυπτός = hidden; παράταξις = the arranging side by side of corresponding parts; ἰδιός = own; ξένος = foreign; ἄλλος = other; πανταχοῦ = everywhere.

a xenotope might find a paratactic partner among the idiotopes of an animal, and the antibodies formed against this xenotope would then be of a paratype that already belonged to the idiom.

Many difficulties are inherent in this concept. The interchangeability of paratactic partners would exclude the existence of random epitypic and paratypic families, as seen by Talmage (101), because this would imply mutual parataxis among the paratopes of the antibody molecules of one animal. But even the assumption that there is only one paratactic partner to every epitope would not rectify the situation. For example, we would have to admit that two mutually paratactic epitypes could both be xenotypes to an animal. This animal, immunized with an antigen presenting one of these two xenotypes, would respond by forming antibodies. If, later, the animal were immunized with an antigen presenting the second xenotype, it would either not form antibodies, or it would form antibodies that would react with antibodies against the first xenotype, because the two paratypes would be mutually paratactic. If the animal were immunized simultaneously with antigens of both xenotypes, it would have difficulties in deciding to which of them it should respond with antibody formation. In all cases the situation would be uneasy.

To avoid these conceptual difficulties, the assumption is made that paratopes and epitopes represent two different classes of stereospecific regions. A paratope is not potentially immunogenic. Paratopes are not epitopes but anti-epitopes; keys are keys, and locks are locks.

IMMUNOLOGICAL TOPOGRAPHY

Though the chemical and physical characteristics of epitopes and paratopes, the nature of paratactic correlation, and the forces engaged in paratactic bonds have been important objects of investigation (20, 49, 50, 76, 82), these problems will not be considered in the present review; they belong to the study of fine structure rather than to topography.

Immunological topography is concerned with the spatial distribution of epitopes and paratopes on molecules and biological structures, and with their classification and enumeration. Single epitopes and paratopes have been variously estimated to extend over a surface area of between 100 and 1000 square angstroms (20, 49, 78). The total surface area of the macromolecular components of the human body is of the order of a square kilometer. On small protein molecules there could be more than ten epitopes; on larger protein molecules and on the smallest virus particles there could be more than a hundred; larger viruses could have more than a thousand epitopes, and the surface of a cell provides space for more than a million. If two neighbouring epitopes can overlap, these numbers become even larger, but, on the other hand, neighbouring epitopes may often be separated by surface areas which form no part of any epitope. An estimate of the number of epitopes on an antigen particle can be based on the number of antibody molecules that can attach to it. It has been found that at least 4 antibody mole-

cules can attach to one RNase molecule, at least 8 to an albumin molecule (60), and over 200 to a hemocyanin molecule (58), whereas more than 1500 antibody molecules have been shown to combine with one T4 bacteriophage particle (61). These experimental findings indicate that epitopes occupy a very large part of the surface area of antigens, and that all antigen particles and molecules are clusters of epitopes. This evidence is the more remarkable since antibody molecules attach only to xenotopes; the estimates based on such evidence do not, therefore, include pantachotopes and other epitopes that are idiotopes to the immunized animal.

There is much evidence indicating that there are usually two paratopes of the same paratype on an antibody molecule (31, 59) which is often pictured as a cigar-shaped structure carrying a paratope at each of its ends. On this molecule there would also be room for perhaps fifty epitopes since the two paratopes occupy only a few per cent of its area. Circulating antibody molecules, however, are heterogeneous, not only in size, but also with respect to several different types of properties (55, 68, 69, 85, 86, 98, 99, 100). Porter (79, 80, 81) has shown that antibody molecules can be fragmented into pieces, some of which retain an intact paratope. It seems reasonable to imagine that such pieces could be incorporated in other structures, such as a cell surface or a permease molecule (63), and that paratopes may be associated with immune functions other than those of circulating antibody molecules.

Do normal gamma globulin molecules present paratopes? Gamma globulin molecules have not been found to possess properties that are not shared by antibody molecules. It is, therefore, usually assumed (11, 101) that normal gamma globulin molecules represent paratypes that are paratactic to unknown xenotypes. It is known that intensive immunization can induce an animal to replace virtually all of its gamma globulin molecules by detectable antibody molecules. It seems likely, therefore, that normal gamma globulin molecules belong to the immunological system, and that they are produced by the same mechanism and present the same structural features as antibodies. The assumption that normal gamma globulin molecules carry paratopes has quantitative consequences because of the large number of paratypes they must represent.

PARATACTIC PERMUTATIONS

Antigen particles that carry between a few and a few million epitopes each, and serum containing gamma globulin molecules each carrying two paratopes of the same paratype, are the essential components in most serological reactions.

Among the epitopes presented by an antigen, a host animal distinguishes between idiotypes and xenotypes. Burnet (11) suggests that there are many more idiotypes than xenotypes among the many epitopes that a protein antigen presents to the host. If this were so, we might conclude, by assuming that protein antigens are random samples of epitopes, that the idiotypes of one animal constitute a large fraction of the epitypic universe. But the close

packing of an antigen particles with antibody molecules in antibody excess suggests that a very large part of the surface of any random antigen particle is made up of epitopes that are xenotopes to a host animal. It seems more likely, therefore, that the idiom of an animal contains only a small fraction of the epitypic universe. This implies that the number of different paratypes which an animal can make is far larger than the number of idiotypes it harbours. If the idioms of animals of different species were each a large fraction of the epitypic universe, then they could hardly be random samples out of this universe. Otherwise, the idioms of a small number of different species would, together, nearly exhaust the epitypic universe and two antigens that cross-react in one species because of a common xenotype would be unlikely to cross-react in another species. In Lederberg's propositions for an elective theory of antibody formation (57) it is assumed that cellular mutations leading to the formation of antibody molecules of paratypes that are paratactic to idiotypes will be followed by the suppression of such mutants. If idiotypes were more frequent than xenotypes the majority of mutants would have to be suppressed.

What are the total numbers of epitopes and paratypes that exist? Rough estimates of the order of magnitude of the epitypic universe have varied from less than 250 (84), through 10,000 (11), to "almost" infinite (101). Similar suggestions as to the total number of different paratypes that an animal can construct range from 5000 (101), through 50,000 (43) and 1,000,000 (48), to potentially infinite (63). An approach to these questions can be based on the frequency of cross-reactions, on the magnitude of serological titres, and on the lower limits of detectable antibody concentrations.

Two antigens may show cross-reaction if they present epitopes of the same epitope or of related epitopes. Cross-reactions may be discussed in connection with the proposal by Talmage (101), that the existence of epitypic and paratypic families of random membership would permit an animal to develop a large number of serological specificities with a comparatively small number of paratypes. This proposal makes it unnecessary to assume that the total number of paratypes of an animal equals the total number of xenotypes. If the epitypic universe consists of A epitopes, with average epitypic families of a epitopes, and the paratypic universe of P paratypes, out of which sets of p random paratypes constitute paratypic families, the probability that a random epitope will be a paratactic partner of a random paratope is $a/A = p/P = \alpha$. Talmage has suggested $P = 5000$, and $p = 50$.

If, in an animal, all paratypes showing parataxis with its d idiotypes were removed, the number of remaining paratypes available to the animal would be reduced to $P e^{-\alpha d}$. Even a low estimate of the size of an idiom, such as $d = 500$, combined with Talmage's suggestion of $\alpha = .01$, would leave the animal with less than 1 per cent of the paratypic universe, and with each average paratypic family reduced to less than 1 per cent of its original size. Disregarding the idiotypes, the probability that two random xenotypes are related, i.e., that there exists a paratype to which both these xenotypes are paratactic, is

$1 - e^{-\alpha p}$. Thus, two antigens each exhibiting m xenotypes would, on the average, cross-react to the extent of $100 \alpha m$ per cent (for small values of αm).

Let us imagine two rabbit sera, against a hemocyanin and against a bacteriophage. A hemocyanin molecule carries hundreds of epitopes which probably include several epitypes since the molecule must present different features along its surface. For bacteriophage T4, at least six xenotypes to rabbits have been demonstrated (36), and it probably carries many more among its more than one thousand xenotopes. Antisera against hemocyanin can easily reach a homologous titre exceeding one million (1). Antisera against bacteriophage T4 can also reach a homologous titre exceeding the least observable titre by a factor of a million (47). Even a 1 per cent cross-reaction only would, in these cases, correspond to heterologous titres of 10,000 which obviously do not occur. Though extensive cross reactions have been observed in other systems (23), the estimate $\alpha = .01$ seems far too high, and if an average family size of 50 paratypes is retained, the total number of paratypes would have to be far larger than 5000. Differences in combining power between members of a paratypic family do not seem to help since paratypes of low combining power would contribute little to high specific titres. It seems more realistic to assume that the membership of a paratypic family is not random but that there is a structural similarity between members which finds its expression in a range of different combining powers and accounts for avidity differences. In that case, the members of a paratypic family would not, at the same time, be members of many other families, and the total number of paratypes would have to be of the same order as the number of epitypes. Jaroslow & Quastler (45) have assumed that there is a dominant paratype to each epitope, and that a homologous reaction between these two can be experimentally distinguished from lesser cross-reactions. Observing about 50 homologous reactions and 2 major cross-reactions in a system of 8 antigens from different phyla, and 6 antisens, Quastler tentatively estimates the number of epitopes in the epitypic universe to be of the order of 1000.

Two other arguments appear to require much larger estimates. One is the argument of the rising titre: for many antigens, the titre of the serum of an animal can increase by a factor of a million after immunization. If the concentration of an average paratope can rise by a factor of a million within an almost fixed total globulin concentration, there are more than one million paratypes. The other is the argument of the detectable antibody concentration: antibody concentrations exceeding 10^{11} paratypes per ml. are serologically observable. Therefore, normal serum, containing a total of 10^{17} paratypes per ml., would show a good titre against any antigen, if all paratypes were equally represented and if there were far less than one million paratypes.

Both of these arguments fail if, as postulated by Talmage (101), the overwhelming majority of normal globulins are antibodies induced by fortuitous exposure of the animal to common environmental antigens in food and bacteria. If this were so, practically all 10^{17} paratypes in 1 ml. of normal serum could represent only a few, say, 1000 paratypes. But then each paratype

would, on an average, be present in a concentration of 10^{14} paratopes per ml.: there would be a very large number of common environmental antigens against which normal sera would show good titres.

THE ANTIBODY-FORMING SYSTEM

An antibody-forming system has a history. A general theory of antibody formation must deal with problems of development and of learning from experience, as expressed in such terms as tolerance, avidity increase, booster response, anamnestic response, and immunological memory. A general theory must be compatible with special theories concerning parts of the system, such as the differentiation of the cells involved and the mechanism of protein synthesis, and it must fit into an even more embracing picture of the total immune response of an animal to foreign invasion.

A functioning antibody-forming system can be regarded as a set of many parallel linear stimulus-reaction chains (22) comparable to a typewriter: we are confronted with questions of choice among such parallel chains, with recognition and maximal complexity. Each linear chain contains a pathway from the entry into the circulation of a particular xenotope to the exit of induced paratopes released by cells into the circulation. A chain may branch off into side tracks: at various points there may be a certain probability that the stimulus proceeds along a channel leading to a different type of response. Also, circular chains may develop; for example, by permitting paratopes, representing the response, to furnish the stimulus in a new cycle. The central operation within each chain is the translation of a xenotype into a paratype, and there is one precise moment which separates the entry problems from the exit problems. This is the paratactic moment, or the moment of recognition. The entry problems are concerned with the events preceding this moment, the exit problems with the mechanism that leads to mass production of antibody molecules of the recognizing paratype. In selective theories, the paratactic moment occurs when the xenotope is recognized by a preformed paratype of which manifold copies are to be made, whereas in the direct template theory the paratactic moment coincides with the moment at which the very first antibody molecule of a new paratype comes into existence at the xenotopic template.

THE AXIOM OF BURNET, AND THE DIRECT TEMPLATE HYPOTHESIS

The axiom of Burnet is crucial in immunological theory: any animal must be capable of discriminating between self and not-self.

Since epitopes are, by definition, recognized by paratopes, an animal must, to distinguish idiotypes from xenotopes, possess a set of paratypes paratactic either to all its idiotypes (self-recognition) or to xenotypes (not-self-recognition). The first possibility is unlikely. Why should idiotypes and paratopes recognize each other, if the only action upon recognition must be to leave each other in peace? In order to obey the axiom of Burnet an animal must, therefore, prior to xenotopic invasion, have established an army of

paratopes that can recognize xenotopes: the paratypic dictionary is a paraxenotypic dictionary, a *Fremdwörterbuch*. But, already knowing how to make these paratopes without xenotopic assistance, the animal need not develop a method of making paratopes on xenotopic templates: the axiom of Burnet makes any direct template hypothesis superfluous.

RECENT THEORIES OF ANTIBODY FORMATION

The following considerations do not take into account all theories and suggestions concerning antibody formation that have been proposed in recent years (14, 19, 26, 27, 35, 51 to 54, 65, 66, 67, 74, 91 to 95), and the omission of valuable ideas may, therefore, limit the relevance of the discussion.

The theories considered are the natural selection theory of Jerne (1955), the clonal selection theory of Burnet (1957, 1958, 1959), the nine propositions for an elective theory of Lederberg (1958, 1959), the antigen-capture model of Monod (1959), and the coated-cell theory of Boyden (1960).

The theories of Burnet and Lederberg are similar; they both postulate that a given antibody-producing cell is genetically competent for making antibody molecules of only one or a few paratypes. The other theories do not contain this restrictive genetic element. The theories of Jerne and Boyden are compatible in postulating that the antigenic stimulus is mediated by preformed antibody molecules returning from the circulation to antibody-producing cells. Monod's model allows clones of cells of increased competence for making antibody molecules of a particular paratype to arise by a non-genetic mechanism. Contrary to the others, Monod and Boyden, moreover, require that the antigen enters the antibody-producing cells and takes part in intracellular processes, as is also envisaged in the direct template theories (6, 14, 41, 42, 51, 64, 75, 77).

The natural selection theory (48) has introduced a new concept into immunological speculation: (a) an animal contains a paraxenotypic dictionary which is different from its idiom; (b) this dictionary is established by a random element and made adequate by a suppression element; and (c) the system functions by reproduction of selected dictionary units and not by the regeneration of lost units. These elements, which do not occur in Ehrlich's side-chain theory (28, 29, 30), are adhered to in all of the more recent selective theories. The differences between these theories concern the location of the dictionary, and the identification of the elements of randomness, suppression, and reproduction. The random element is necessary to account for paratypes against xenotypes of which the animal or its ancestors have had no experience. The dictionary units representing each paratype must be sufficiently numerous and accessible to permit any invading xenotope to consult the entire paratypic dictionary within a reasonable time. This implies, if the dictionary is to be complete, that the full set of all possible paratypes is small compared to the number of dictionary units available to the animal.

The natural selection theory locates the units of the dictionary where paratopes are known to occur: on circulating globulin molecules. It identifies

the random element with some degree of freedom in the assembly of a short amino acid sequence, responsible for the paratype, in spontaneous globulin synthesis. It identifies suppression with the removal of undesirable paratopes by idiotopes. Spontaneous globulin synthesis and suppression presumably occur mainly in embryonic life. Reproduction is envisaged as the reproduction of paratopes after the return of globulin molecules from the circulation to competent cells, and as the multiplication of these cells. Antibody formation is the preferential reproduction of the paratopes of circulating globulin molecules selected by xenotopes, whereas the formation of normal gamma globulin may be the non-preferential reproduction of non-selected paratopes of circulating globulin molecules returning to competent cells. This idea of the reproduction of paratopes presented by circulating globulin molecules returning to antibody-forming cells has generally been considered unacceptable.⁴ Talmage (99) and Burnet (9) have stated that the only tenable form of the natural selection theory must involve the existence of cells each responsible for producing antibody molecules of only one genetically determined paratype, and the selective stimulation of such cells by paratactic xenotopes. This implies that antibody molecules, once produced and released into the circulation, are all definite end-products that no longer have a function in subsequent antibody formation.

Burnet (9, 10, 11) has formulated the clonal selection theory which locates the paratypic dictionary in cells, each of which is genetically capable of producing globulin molecules of one paratype only. The consultation of this cellular dictionary by xenotopes is made possible by the display, on the surface of the cells, of paratopes representing the paratype that the cell is prepared to make. Parataxis at the cell surface may call forth several types of response, the most important being the conversion to plasma cell and the liberation of antibody globulin.

The random element is identified with the random process *par excellence* in biology: cellular mutation. Suppression is the elimination, during a sensi-

⁴ Because equivalent to a replication of protein. The reproduction of selected paratopes, though not necessarily involving direct protein replication, appears to violate Crick's (105) *Central Dogma*: that information concerning amino acid sequence, after passing into protein, cannot get out again. If paratype is determined by amino acid sequence, then antibody formation shows that heterogeneous foreign proteins can deliver messages leading to the synthesis of unique amino acid sequences. The natural selection theory proposes that such a message becomes readable to antibody-forming cells only after relay through a preformed paratope, i.e., through the mediation of a member of a structurally unique class of molecules, selected and activated by parataxis. The clonal selection theory accepts a relay of the message through a selected paratope, but avoids a conflict with the *Central Dogma* by assuming that this paratope is fixed to a cell that is already genetically equipped with the corresponding paratypic information. If a postulate were added to the natural selection theory, restricting the reproduction of any selected circulating paratope to cells specializing in its particular paratype, then the two theories would become virtually identical.

tive phase, of cell mutants displaying paratopes that are paratactic to circulating idiotopes. The elimination of such clones is presumably completed in embryonic life. The clonal selection theory permits a reasonable interpretation of a large number of immunological phenomena (11), and it has the advantage that an experiment is easily conceivable, which could disprove it: if a single cell could be shown to produce antibodies against two or more randomly chosen xenotypes. The clonal selection theory would then have to adopt the additional assumption that stimulated cells can exchange genetic information.

Lederberg (56, 57) has formulated an elective theory in nine propositions which add some special as well as general features to the original clonal selection theory. He specifies (i) that a given paratype represents a unique amino acid sequence, (ii) derived from a unique nucleotide sequence in a segment of the chromosomal DNA of an antibody producing cell; (iii) in the precursors of such a cell, this segment is subject to a high rate of mutation, (iv) permitting random DNA assembly in the globulin gene during certain stages of cellular proliferation; (v) each cell, before maturation, spontaneously produces a small amount of antibodies, or paratopes, of its own genotype, that can mark the surface of the cell which (vi) passes through an immature phase in which it is hypersensitive and will be suppressed if it experiences a paratactic encounter; (vii) parataxis stimulates mature cells to accelerated globulin synthesis and cytological maturation to plasma cell; (viii) stimulated cells are genetically stable or subject to a medium mutation rate, (ix) forming clones that tend to persist after the disappearance of the antigen. Lederberg thus also identifies the dictionary units with cells that display their restricted genetic capabilities. His proposals deal more elegantly with the suppression element, permitting it to function continuously throughout life: cell mutants exhibiting inadequate paratopes will be suppressed by idiotopes during their hypersensitive phase, and acquired tolerance remains effective during the presence of circulating tolerated xenotopes. The proposal (viii) permits mutation of the descendants of stimulated cells, but it is not clear whether such descendants pass through a hypersensitive phase. If they do, many will be eliminated by xenotopes; if not, undesirable mutants will escape the idiotopic purge. Lederberg suggests that, if a single cell can be shown to make antibody molecules of two or more random paratypes not likely to occur among the few paratypes that can be genetically provided for, then a most powerful elective theory could be generated by shifting the basis for paratypic expression to nucleotide sequences of ribosomal RNA; autonomous variation of ribosomes would allow for any imaginable multiplicity of paratypes, and a selection theory *en miniature* would operate within the narrow confines of a single cell. This idea seems to be incompatible, however, with Burnet's axiom. Random sets of increasing numbers of paratypes will, with increasing probability, include paraidiotypes; if cells representing such sets are susceptible to paratactic stimulus of their surface paratopes, then an increasing majority of the cells would either be suppressed by idiotopes during

the hypersensitive phase, or produce antibody molecules of forbidden paratypes. Thus, both the clonal selection theory of Burnet and the modifications proposed by Lederberg, are equally vulnerable to an experimental demonstration of the production, by single cells, of antibodies against two or more random xenotypes.

Results of single cell experiments are now available (2, 70, 71, 104). Those presented by Attardi, Cohn, Horibata & Lennox (2) do not support Burnet's and Lederberg's theories; they show that single cells can produce antibodies against at least two randomly selected xenotopes. Rabbits were hyper-immunized by injection, into the footpads, of the serologically unrelated bacteriophages T2 and T5. Washed cell suspensions from popliteal lymph nodes, mixed with bacteriophages T2 and T5, were dispensed into microdrops, and incubated. Among 925 single cells, each residing in a microdrop, 95 produced antibody in sufficient quantity to show significant inactivation of bacteriophage. Antibodies against either T2 or T5 was formed by 74 cells, whereas 21 cells produced both. Incidentally, microscopic inspection showed that these antibody releasing cells included stem cells, lymphocytes, and plasma cells. This observation is an important contribution to the cytology of antibody formation (11, 32, 33).

Since bacteriophages present, to a rabbit, many unrelated xenotopes, only some of which occur on structures (7) that are vulnerable to antibody, the results obtained by the Cohn-Lennox group reflect only part of the antibody-forming capacity of the cells observed. It does not seem likely that this demonstration of the production of two paratypes by one cell can be interpreted as the selection of mutants, by the second xenotope, among clones derived from cells selected by the first. If single cells, in their DNA or RNA, contain pre-existing information that enables them to produce antibodies of two random paratypes, then each must represent a large fraction of the paratypic dictionary; each cell becomes a glossary. But, as set out above, a cell representing a random glossary must not invite stimulation by advertising all its paratypic markers because they include paraiddiotypes. The cell cannot, therefore, be selected by xenotopes; xenotopes must consult a paratypic dictionary elsewhere.

Monod (63), illuminating the similarities and differences between antibody formation and enzyme induction in bacteria, has suggested the antigen-capture model of antibody formation. According to this model, the induction of a cell to antibody formation is mediated by permease molecules functioning at the surface of an inducible cell by facilitating the entry of xenotopes, in analogy with the situation described in enzyme induction (16, 17, 18, 62, 72, 88). Xenotopes that have entered the cell induce both the production of antibody molecules and the production of permease molecules which, in turn, permit more xenotopes to enter, until the cell is fully induced. Induction thus has the effect of increasing the sensitivity of the cell to low concentrations of the inducer. Monod's model does not regard the inducible cells as genetically determined units of a purged, random paraxenotypic dictionary as the clonal

selection theory does. On the contrary, the cell population is regarded as a genetically homogeneous population that utilizes the permease system to obtain a memory effect; the induced permease molecules are distributed among the descendants of induced cells which thus represent genetically unchanged clones exhibiting greatly increased sensitivity to a later homologous xenotopic stimulus. Uninduced cells must either possess innumerable genetically preinscribed potentialities among which penetrating epitopes can make a selection, or the cells must possess the general potentiality of accepting epitopic instruction. In the first case, there would be a greater similarity to the induced enzyme system. In the latter case, the model is an important extension of the direct template theory, providing for immunological memory in the absence of antigen. In neither case does it provide for the axiom of Burnet.

Boyden (3) has proposed the coated-cell theory of antibody formation which likewise denies the genetic traits of the clonal selection theory and of Lederberg's propositions, but which combines the main features of the natural selection theory with Monod's model and with a direct template hypothesis. Boyden's theory depends initially, as does the natural selection theory, on the spontaneous production and excretion by specialized cells of globulin molecules of random paratypes. By idiotopic removal, these circulating globulin molecules become a purged, random paraxenotypic dictionary. The novelty of Boyden's theory is the idea that antibody-forming cells adsorb globulin molecules and become coated with them. At the cell surface, these coating molecules play the rôle of receptors, permitting paratactic xenotopes to penetrate into the cell and to participate in antibody synthesis. Most of the antibody molecules produced are released into the circulation but some remain at the surface of the producing cell, facilitating the entry of more xenotopes, until the cell is fully induced and provided with an immunological memory that can be legated to its descendants. The theory thus incorporates all features of Monod's model. Furthermore, Boyden has attempted to account for the intracellular mechanism by which xenotopes direct antibody synthesis. A xenotope that has penetrated into a cell is confronted with an intracellular pool of unstable globulins of random paratypes that are continuously synthesised and broken down at equally high rates, except for those that happen to show parataxis to the xenotope. Since an antibody-forming cell must be able to produce a few thousand antibody molecules per second, Boyden's suggestion implies that at least a thousand times all possible paratypic configurations make their momentary appearance in the cell every second. A cell cannot possibly assemble globulin molecules, from amino acids, at this rate. Boyden's intracellular cycle seems feasible only if it represents merely a continuous coiling and folding of globulin polypeptide chains in the vicinity of xenotopes, until parataxis is achieved: but this is closely similar to the direct template mechanism.

Both Monod's model and Boyden's theory, by requiring the xenotope to enter the antibody-producing cell, can hardly avoid a conflict with Burnet's

axiom. If the whole antigen particle, or a part of the antigen particle, is permitted to enter the cell, then the effect of any extracellular recognition system becomes undone, because the particle will carry both idiotopes and xenotopes, as well as cryptotopes, into the cell. An intracellular discriminating mechanism is then needed but difficult to imagine. If intracellular units exist that can recognize xenotopes, they may need stimulus but not instruction from a xenotope template since the information is already inherent in their ability to recognize. On the other hand, an isolated xenotope cannot enter the cell, because a xenotope is not a particle, but an image carried by a particle; is it not more reasonable to let the paratope of a globulin molecule convey this image into a cell? Why, if a protein template is at all desirable and acceptable, cannot a cell utilise a double-template mechanism, letting the paratope of a returning antibody molecule serve as a template for the construction of a self-made master-template, more suitable than errant foreign matter for imprinting a paratype on new antibody molecules?

THE RETURNING ANTIBODY MOLECULE

Speculating about the evolutionary development of the phenomenon of antibody production, and the consequences of Burnet's axiom for simple metazoa that possess only a few phagocytes dealing with nutrition and defence, Boyden (3) has put forward the following considerations. To recognize foreign matter, these phagocytes must be display paratopes. Each cannot represent one paratype, because there are not enough cells, and because each cell is able to deal with a large variety of foreign matter. Each cannot produce a large set of random paratopes for display because the paraidiotopes which these would frequently include would make the cell inadequate. It is, therefore, reasonable to imagine that the phagocytes are coated with paratopes produced elsewhere; if other cells produce and liberate a population of molecules of random paratypes, idiotopes could purge this population and an adequate set would remain either for the coating of phagocytes or for the coating of foreign matter. It is easier to envisage the elimination of many inadequate molecules than of many inadequate cell variants.

Simple animals might take advantage of developing only a limited dictionary, including paratopes directed against pantachotypes not occurring in their idiom. This simple expedient would not work in animals possessing extensive idioms, and the need for paratopes against all xenotypes may be the reason why vertebrates have found it necessary to bathe their tissues in a pool of circulating globulins.

Boyden & Sorkin (4) have shown that normal rabbit spleen cells actually become coated with antibodies when suspended in serum *in vitro*; these cytophilic antibodies are not removed from the cell surface by washing, and they are capable of mediating the specific adsorption of antigen to the cells. There is thus experimental evidence for the return of at least a small fraction of circulating antibody molecules to lymphoid cells, at the surface of which they can attract paratactic xenotopes. In regarding this return of antibody mole-

cules to lymphoid cells as a crucial step in the antibody-forming mechanism, Boyden is in agreement with the natural selection theory. The proposal of the returning antibody molecule does not imply that all globulin molecules have this function. Antibody molecules are known to be heterogeneous and their large majority may not possess the property which permits them to reproduce their paratype by entering this pathway of return. They may exercise other functions, for which their recognizing paratopes make them suited, such as the elimination of foreign matter.

Experiments which do not seem to favour the role of returning antibody molecules in antibody formation are those which indicate (a) that enhanced capability of forming antibodies of a particular paratype can be transferred by cells from immunized animals to normal, tolerant, or incapable animals (12, 40, 89); (b) that antibody-forming cells can, perhaps, be stimulated by antigen *in vitro* (12, 34, 37, 38, 39, 44, 83, 90, 96, 97, 102); and (c) that the transfer of serum from immunized animals does not enhance but depresses antibody formation by a normal animal of the same species. Experiments of type (a) show that it is an advantage to the animal to have cells that have already been fully induced and are more readily competent. Here, as in experiments of type (b), the possibility remains that such cells are already coated with globulin molecules that can accept the antigen stimulus. Also, in such experiments, the enhancing influence of the presence of autologous serum is often noted. The experimental evidence of type (c) has been regarded as a disproof of the natural selection theory, because an animal would not be able to distinguish between its own globulin and that of another member of the same species (99). But Stevens & McKenna (97) have shown that the antibody-forming response of animal spleen tissue stimulated with antigen *in vitro* could be enhanced tenfold by the presence of normal autologous serum, whereas isologous serum had no enhancing effect. Also, the presence of different, genetically determined allotypes on circulating globulins of different individuals of the same species has been demonstrated by Oudin (24, 73). The animal receiving isologous serum recognizes these allotypes and may channel the isologous antibody molecules along a different path.

The extraordinary recognizing faculty of animals has also been brought out by the experiments of Cinader & Dubert (15), which have recently been confirmed with even more clear-cut results by Boyden (5). They show that a tolerant animal continues to distinguish the xenotopes of a tolerated antigen from its own idiotopes; the animal will produce antibodies against a hapten coupled to its own albumin molecules, but will not produce antibodies against a hapten coupled to a foreign albumin molecule to which it is tolerant. The tolerant animal must, therefore, possess molecules which recognize the tolerated protein, and which prevent this protein, as well as the coupled hapten, from being channelled into the pathway leading to antibody-formation against the hapten. Bussard (13) has recently demonstrated the existence in tolerant animals of such circulating molecules which attach to tolerated protein but which are not detected in usual antibody reactions.

No general theory can possibly account, at present, for all the innumerable complexities of antibody formation. Can the striking increase in avidity, or combining power (46, 47, 68, 69), of antibody during immunization be more satisfactorily explained by the selection of cellular mutants, or (57) by the release of early inadequate antibody from suppressed immature cells, or by the preferential selection by xenotopes of better fitting paratopes of globulin molecules in the feed-back mechanism of the natural selection theory?

Is the partial denaturation of antibody molecules selected by xenotopes, and the fixation of complement, involved in the antibody-forming stimulus chain? How to explain the important observations of Dubert (25, 26) concerning the specificity of the secondary response, showing that an animal primarily immunized with protein molecules carrying a coupled hapten, respond with antibody formation against this hapten when secondarily stimulated with the protein alone? Any explanation would seem to require that it is the same cell which produces globulin molecules of the antiprotein and the antihapten paratype in this secondary response.

In view of the difficulties facing the genetic selection theories of Burnet and Lederberg, as well as any direct template theory, the mediation of antibody formation by circulating antibody molecules returning to cells still seems a hypothesis that is worth considering.

I shall conclude with Melvin Cohn's (63) question: why do antibody molecules present two paratopes of identical paratype? Whenever, in biology, we come across molecules that carry the same information *in duplo*, it is reasonable to think of reproduction. The famous example is the DNA molecule. The antibody molecule is, of course, very different. Though there is doubt, it is generally thought of as a rod carrying identical paratopes at each of its ends. Where else, in the universe, do we find sticks carrying the same monogram at both ends? I can think of only one example: the system of rods in a typewriter, which mediate between the fingers of a typist and the formation of printed letters in selected stimulus chains.

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